UNIVERSITÉ FRANÇOIS RABELAIS TOURS

École Doctorale SANTÉ, SCIENCES, TECHNOLOGIES

Institut de Recherche sur la Biologie de l'Insecte

THÈSE présentée par :

Clément VINAUGER

soutenue le : 21 Octobre 2011

pour obtenir le grade de : Docteur de l'université François - Rabelais

Discipline/ Spécialité : Sciences de la Vie

APPRENTISSAGE ET MÉMOIRE CHEZ LES INSECTES VECTEURS DE MALADIES HUMAINES

THÈSE DIRIGÉE PAR :

M. Lazzari Claudio	Professeur, Université François Rabelais, Tours

RAPPORTEURS :

M. Giurfa Martin	Professeur, Université Paul Sabatier, Toulouse
M. Lehane Michael	Professeur, School of Tropical Medicine, Liverpool, Angleterre

JURY :

M. Casas Jérôme	Professeur, Université François Rabelais, Tours
M. Giurfa Martin	Professeur, Université Paul Sabatier, Toulouse
M. Lazzari Claudio	Professeur, Université François Rabelais, Tours
M. Lehane Michael	Professeur, School of Tropical Medicine, Liverpool, Angleterre
M. Pereira Marcos	Professeur, Universidad Federal do Minas Gerais, Brésil

Remerciements

Pour commencer, je me dois de remercier mon directeur de thèse Claudio Lazzari. Je le remercie d'avoir été un directeur de thèse patient, disponible, encourageant et motivant. Merci pour cette thèse, pour ce sujet qui m'a passionné et m'a beaucoup apporté scientifiquement et humainement, et merci pour les occasions de voyager. Je te remercie de m'avoir permis de participer au cours d'écologie sensorielle de Lund, pour les tous les congrès et pour le stage au Brésil. Merci également pour "l'effet Lazzari", pour ta *geekness* latente et pour l'approvisionnement en herbe (Je parle bien entendu de Yerba Maté).

Je remercie Martin Giurfa et Michael Lehane pour l'intérêt qu'ils ont porté à mon travail et pour avoir accepté d'en être les rapporteurs. Je remercie également Jérôme Casas et Marcos Horacio Pereira d'avoir accepté d'être examinateur de ma thèse.

Un grand merci à Jean-Paul Monge pour m'avoir accueilli au sein du l'IRBI, et je remercie également le Ministère de l'Enseignement Supérieur et de la Recherche pour la bourse doctorale qui m'a été attribuée. Un grand merci à Marcos, sa famille et son équipe pour l'accueil chaleureux qu'ils m'ont réservé à Belo Horizonte, pour m'avoir fait découvrir leur pays et leur culture. Merci en particulier à César pour toute l'aide qu'il m'a apporté.

Un grand merci à Carole Labrousse pour sa gestion de l'élevage de punaises, son soutien et son aide dans la préparation des expériences et pour ses encouragements. Merci également pour toutes les conversations et la bonne humeur.

Merci à Bruno et Guy pour leur soutien technique, leur créativité et leur disponibilité sans failles.

Merci à l'ensemble du groupe "punaises" de l'IRBI, Dominique, Nadine, Teresita, Chloé et maintenant "Mordi" pour les discussions, débats et réflexions (scientifiques ou non d'ailleurs).

Merci à tous ceux qui sont passés par le bureau I1330, Maurane, Hadrien, Mélanie, Baptiste, Marion, Sébastien, Raquel, avec une mention spéciale pour Aurélie avec qui j'ai réalisé mes stages de master et Fabienne pour les conversations scientifiques, pour sa gentillesse et pour son soutien sans failles. Bien conscient d'en dire trop ou pas assez, je tiens néanmoins à rétablir la vérité sur un point précis à leur sujet : celle qui parle le plus n'est pas celle que l'on croit.

Un grand merci à Laura pour le stage effectué avec moi sur la première partie de la thèse. Merci pour ton travail soigné et rigoureux, pour la bonne humeur et la motivation à toute épreuve.

Merci aux thésards du labo qui m'ont rendu la vie là bas agréable.

Manh et Odette pour la bonne humeur et les repas à thème, Gaelle et Vincent pour les sorties fluviales et cinématographiques, Marjorie pour les gâteaux avec les raisins secs dedans, Mélanie pour les recettes improbables, Julien, Aurélien et Germain pour leurs encouragements, leur soutien et aussi pour le style et Denis et Sylvain pour toutes les discussions.

Merci également à tous ceux qui ont participé à l'organisation du congrès SERL. Et en ces temps de rédaction, un grand merci à Jonathan et Elfie qui m'ont particulièrement bien aidé à affronter cette épreuve.

Je remercie également tous les membres de l'IRBI qui ont rendu ma thèse plus vivante et merci à celles et ceux qui m'ont apporté une aide logistique ou administrative précieuse (Sonia, Christine, Nadine, Maryse, Séverine D et Séverine P, Hélène, Jean-Philippe et *last but not least* Simon).

Je remercie les *aficionados* avec qui j'ai eu le plaisir de jouer au poker, Sylvain, Guirec, Fabienne, Franck, Rebecca et Jérémy.

Merci à tous mes amis "hors-thèse", pour me rappeler que la vie ne s'arrête pas à la paillasse et pour tout ce qu'ils m'ont apporté.

Merci à Julien Fouché, dit "Fouch", globe trotteur insaisissable, pour le soutien, l'encouragement, l'émulsion, la créativité, la philosophie au quotidien, la cuisine expérimentale, sa culture et ouverture d'esprit. Rare le gars, rare.

Merci aux "wookiees", Dim, Ronan, Eric et Julien pour l'amitié, la musique, les bières et votre passion pour les T-shirts. *Tching-Tchong* messieurs. Bravo à Romain pour la maîtrise de ses narines et à Rudy pour les encouragements. Merci également à tout ceux qui ont vécu ou connu la Kip's, Utopsia, Alf et les dindons, carte blanche, les fabulous glander's, Shelter et ATC.

Merci à tous ceux qui ont toujours été là et qui le seront encore pendant longtemps : Yann, Rémi, Philippe, Bertrand, Guillaume et tous ceux que je n'oublie pas, à qui je pense très fort, mais dont la liste exhaustive ferait probablement tripler le nombre de pages de ce document. Que ceux-ci me pardonnent.

Je tiens également à remercier Dominique B., musicien, philosophe et dentiste, pour avoir pris soin de ma santé bucco-dentaire tout au long de ma thèse. Je le remercie également pour les discussions musico-centrées.

Merci à Damien Schmitt pour m'avoir souvent aidé à relativiser. Ceux qui comprennent, savent.

Je remercie toute ma famille : grands-parents, parents, sœur, oncles et tantes, cousins et cousines, pour leur soutien, leurs encouragements, leur patience et leur compréhension.

Merci à Lionel, Marie-Paule et leurs parents, frères, sœurs et enfants pour tout ce qu'ils m'ont apporté, pour leur accueil et leurs encouragements pour la thèse.

Enfin, un ultra merci à Chloé, qui me supporte au bureau, en manipe, aux élevages, en congrès et dans la vraie vie tout en parvenant à chaque fois à trouver les mots pour m'encourager, me soutenir et m'aider à avancer. Du fond du cœur un grand GRAND merci à toi pour tout ce que tu es et tout ce que tu m'apportes.

Résumé

En permettant aux animaux de faire face à des environnements variables, l'apprentissage et la mémoire contribuent à l'optimisation de leur fitness, en leur permettant d'extraire et d'utiliser des informations, de façon à réduire l'incertitude associée à des environnements imprévisibles. Parmi les insectes, la drosophile et l'abeille domestique sont considérés comme des modèles classiques pour l'étude de l'apprentissage et de la mémoire. Les travaux réalisés sur ces derniers ont apporté une quantité considérable d'informations concernant les bases génétiques, neurobiologiques et moléculaires de ces processus, et ont permis de rendre compte du niveau de complexité des capacités cognitives des insectes. Cette somme de connaissances fondamentales acquises chez ces insectes contraste étonnamment avec le faible niveau de connaissance concernant la cognition des espèces impliquées dans des problématiques qui touchent la santé humaine et animale. Pourtant, il est largement admis que l'étude détaillée des capacités cognitives des insectes vecteurs de maladies constitue un aspect prioritaire pour la compréhension de leurs adaptations à la vie hématophage, de leur importance vectorielle, ainsi que pour le développement de nouveaux outils pour leur contrôle. Les travaux réalisés à ce jour chez les vecteurs, principalement chez les moustiques, ont été menés dans des contextes naturels ou peu contrôlés et ne proposent donc pas de démonstration formelle d'apprentissage. Le principal objectif de ce travail de thèse est de proposer un cadre expérimental contrôlé permettant de mettre en évidence et caractériser les capacités d'apprentissage chez la punaise hématophage *Rhodnius prolixus*. À la différence des moustiques, les caractéristiques biologiques de cette punaise hématophage, responsable de la transmission de la Maladie de Chagas en Amérique Latine, permettent l'adaptation de protocoles expérimentaux largement validés chez les drosophiles et l'abeille domestique. Nos résultats montrent dans un premier temps que ces insectes sont capables d'apprendre et d'associer la présentation d'une même odeur dite neutre (l'acide lactique), c'est-à-dire qui ne provoque ni attraction ni répulsion lorsqu'elle est présentée seule, avec soit la possibilité d'obtenir une récompense (un repas sanguin, conditionnement appétitif), soit avec la possibilité de recevoir une punition (un choc mécanique, conditionnement aversif). Nous avons également montré que l'apprentissage et la mémoire sont également impliqués dans le choix des hôtes. Les insectes ont en effet associé la présentation d'un choc mécanique avec le complexe d'odeur d'hôtes naturels, biaisant leur préférence lors d'un test de choix réalisé après l'entraînement. Dans un second temps, nous avons adapté à notre modèle d'étude le paradigme de conditionnement de la réponse d'extension du proboscis, développé chez les modèles classiques, ce qui a permis la caractérisation des capacités d'apprentissage, de la durée de rétention à la régulation par des horloges circadiennes. Ces travaux proposent également un paradigme expérimental, reproductible et efficace permettant d'analyser les mécanismes fins qui sous-tendent les processus d'apprentissage et de mémorisation. Dans son ensemble, cette étude apporte la première preuve expérimentale de la capacité d'apprentissage d'insectes vecteurs de la maladie de Chagas et propose des outils expérimentaux et méthodologiques permettant d'améliorer la compréhension des processus associés chez les insectes hématophages en général. Les résultats sont également discutés dans le contexte de la sélection d'hôte et de la transmission des parasites.

Mots clés : Apprentissage, Mémoire, Conditionnement appétitif, Conditionnement aversif, Habituation, Déshabituation, PER, Modulation Circadienne, Chronobiologie, Maladie de Chagas, Insectes hématophages, Olfaction, Thermoperception, Choix de l'hôte, Repas sanguin, *Rhodnius prolixus*.

Abstract

Learning and memory contribute to animals' fitness by allowing them adapting to variable environments. Thses two processes make them able to extract and use information from their environment in order to reduce the uncertainty associated with unpredictible environments. Among insects, fruit flies and honeybees are considered as classical models for the study of learning and memory. The amount of work that has been done on these models provide a considerable amount of information regarding the genetic, neurobiological and molecular basis of these processes and revealed the complexity of insects' cognitive abilities. All this knowledge acquired in model species, contrasts surprisingly with the lack of knowledge available regarding insect species that are involved in animal and human diseases transmission. Yet, it has been aknowledge that the detailed study of vectors cognitive abilities would allow the understanding of their adaptation to haematophagy, of their vectorial importance and provide new tools for diseases control. Up to date, studies focusing on disaese vectors, mainly in mosquitoes, were conducted in natural or not completely controled contexts and thus no clear demonstration of learning and memory is availaible.The main goal of this work was to provide a controled experimental context allowing the strudy of learning abilities in the haematophagous bug *Rhodnius prolixus*. Our results show that these insectes are able to learn to associate the delivery of a same neutral odour either with the possibility to obtain a reward (blood-meal, appetitive conditioning) or with the possibility to receive a punishment (mechanical shock, aversive conditioning). We also showed that learning ans memory are involved in host selection processes. In a second part, we adapted to our biological model the paradigm of proboscis extension response conditioning, which allowed us to analyse and characterize its learning abilities. The maximal retention duration as well as the modulation of learning abilities by circadian clocks were evinced. Taken as a whole, this work provides the first experimental demonstration of learning abilities in Chagas disease vectors and provides experimental and methodological tools ; These latters should allow improving the understanding of the mechanisms that are underlying cearning abilities of haematophagous insects in general. Results are also discussed in the context of host selection and parasite transmission.

Keywords : Learning, Memory, Appetitive conditioning, Aversive conditioning, Habituation, Dishabituation, PER, Circadian modulation, Chagas disease, Host choice, *Rhodnius prolixus*.

Table des matières

Table des figures

Introduction

L'apprentissage et la mémoire peuvent êtres définis comme la modification du comportement d'un animal basée sur des expériences passées et le stockage des informations expérientielles (Lorenz, 1981). En permettant aux animaux de faire face à des environnements variables, ces processus contribuent à l'optimisation de la fitness des animaux qui en sont capables, en leur permettant d'extraire et utiliser des informations, de façon à réduire l'incertitude associée à des environnements imprévisibles.

Parmi les insectes, la drosophile et l'abeille domestique sont considérées comme des modèles classiques pour l'étude de l'apprentissage et de la mémoire. Les travaux réalisés sur ces dernières ont apporté une quantité considérable d'information concernant les bases génétiques, neurobiologiques et moléculaires de ces processus, et ont permis de rendre compte du niveau de complexité des capacités cognitives des insectes.

Cependant, cette somme de connaissances fondamentales acquise chez ces insectes, contraste étonnamment avec le faible niveau de connaissance de la cognition des espèces impliquées dans des problématiques qui touchent la santé animale et humaine. Pourtant, il est admis que l'étude détaillée des capacités cognitives des insectes vecteurs de maladies humaines et animales constitue un aspect prioritaire pour la compréhension de leurs adaptations à la vie hématophage, de leur importance vectorielle, ainsi que pour le développement de nouveaux outils pour leur contrôle.

Malgré un important effort de recherche, notamment chez les moustiques, une révision récente de la littérature, révèle que peu d'études apportent des preuves expérimentales concernant les capacités d'apprentissage des hématophages (Alonso et Schuck-Paim, 2006).

L'objectif de ce travail de thèse est donc de fournir des outils permettant d'évaluer l'importance épidémiologique des processus d'apprentissage et de mémoire chez les insectes hématophages vecteurs de maladies (punaises, moustiques, etc.). Pour ce faire, la réduve *Rhodnius prolixus* (Triatominae : Reduviidae), s'est présentée comme un modèle de choix. En effet, à la différence des moustiques, les caractéristiques biologiques de cette punaise hématophage, responsable de la transmission de la Maladie de Chagas en Amérique Latine, permettent la mise en place de protocoles expérimentaux largement validés chez les drosophiles et l'abeille domestique. Le but des expérimentations réalisées dans ce travail est d'apporter des éléments permettant de confirmer expérimentalement la présence de capacités d'apprentissage chez les vecteurs de maladies. Pour cela, il nous faudra identifier les obstacles à franchir afin d'apporter une démonstration expérimentale claire et reproductible de la capacité des vecteurs à apprendre et mémoriser des informations.

Le manque de données expérimentales est dans certains cas la conséquence d'une méconnaissance des définitions. L'étude de l'apprentissage requiert de se placer dans un cadre théorique exigent et contrôlé. De fait, avant d'aborder les questions posées par l'étude le l'apprentissage et de la mémoire chez les vecteurs de maladies, il parait nécessaire de préciser les définitions des concepts que nous allons aborder ainsi que de revenir sur les méthodologies développées et les connaissances acquises chez les modèles classiques.

L'apprentissage et la mémoire, concepts et définitions

Apprentissage et mémoire.

Pourquoi est-il nécessaire de donner une définition de l'apprentissage et de la mémoire, tels que nous les avons appréhendés dans cette étude ? Tout simplement parce qu'il n'y a pas de consensus atour de la définition de ces processus. Le rôle de la définition est de décrire les principales caractéristiques ou structures d'un concept, notamment pour le délimiter, l'isoler des autres concepts et rendre possible une exploration systématique du sujet (Cohen et Nagel, 1934, p. 232). Il parait de plus nécessaire de trouver un compromis

entre la précision et la souplesse de façon à 1) ne pas exclure de nouveaux domaines d'observation et 2) essayer d'appliquer des définitions de l'apprentissage basées chez les mammifères, chez les invertébrés.

Or, il est étonnamment difficile de définir l'apprentissage d'une façon pleinement satisfaisante. Selon le modèle étudié, la discipline concernée (de la génétique à la psychologie expérimentale en passant par la biologie moléculaire et l'éthologie) et la théorie de l'apprentissage concernée (i.e. Behaviourisme, Cognitivisme, Constructivisme, Humanisme ou Connectivisme), la définition peut varier, écartant ou intégrant certaines formes d'apprentissage.Corning *et al.*(1973a) iront même jusqu'à écrire que la définition de ces phénomène dépend du " *Weltanshauung*", c'est-à-dire de la vision du monde de celui qui les définit.

En 1956, dans un ouvrage intitulé "*Learning and Instinct in Animals*", W.H. Thorpe définissait l'apprentissage comme "*le processus qui se manifeste par un changement adaptatif du comportement, résultant de l'expérience*". Cette définition est basée sur la valeur adaptative de l'apprentissage tandis que d'autres excluent ce critère. Bush et Mosteller (1955), par exemple, considèrent comme apprentissage tout changement comportemental, que ce changement soit adaptif ou non.

Miller (1967) donne une définition plus restrictive, basée sur des travaux réalisés chez les mammifères. Pour lui, l'apprentissage correspond à une augmentation, plus ou moins permanente, de l'intensité d'une réponse en raison d'un renforcement passé de cette dernière. Cette définition exclue donc de fait les phénomènes non-associatifs que sont l'habituation, la sensibilisation et le pseudo-conditionnement. A l'inverse Eisenstein (1967), suite à ses travaux d'enregistrement de ganglions thoraciques isolés de blattes, définit l'apprentissage de la façon suivante :

> "*A system is said to demonstrate learning when its output (Response) to a given test input of (Stimulus) is a function of the total previous input-output pattern of which the test input was a part. That is, a system can be said to have learned if its output to a given test input is a function of the specific input-output pattern to which it has been exposed.*" (Eisenstein, 1967).

En d'autres termes, un système apprend lorsqu'il ajuste sa "sortie" (*output*, qui fait ici référence à la réponse, qu'elle soit comportementale ou non) face à une stimulation, en fonction du pattern temporel de la ou des entrées (stimuli) précédentes (*input*, faisant ici référence aux stimuli, qu'ils soient conditionnels ou inconditionnels). Cette définition permet l'étude d'une plus grande variété de systèmes et n'exclue pas la prise en considération de systèmes simples isolés (préparations anatomiques) ou intacts (invertébrés) comme des préparations adaptées pour l'étude des mécanismes (neurophysiologiques, neurochimiques et évolutifs) qui sous-tendent l'apprentissage tel que défini par Miller.

Corning *et al.*(1973) confirment cette idée que la connaissance des mécanismes de base de l'apprentissage et de la mémoire (tels que décrits en 1973), sont trop limités pour baser la définition de l'apprentissage sur l'exclusion. Ils préfèreront donc, quant à eux, parler de "*plasticité comportementale*" et de la "*plasticité du système nerveux*", tout comme Konorski (1948) et Livingston (1966), ou encore de "*modification comportementale basée sur l'expérience*". Ils s'intéressent donc aux caractéristiques des systèmes qui démontrent 1) une modification comportementale (réponse / output) fonction des événements qui ont précédés ou suivis cette réponse lorsqu'elle fut évoquée par le passé et 2) dont les changements sont supposés êtres régulés par des modifications structurales au sein du système. Ce point de vue tient donc compte de multiples phénomènes : réactions immunologiques, habituation, sensibilisation, pseudo-conditionnement, conditionnement classique, opérant jusqu'aux formes d'apprentissage les plus complexes. Aujourd'hui la définition qui semble être la plus communément admise se réfère donc à un *changement relativement permanant du comportement, résultant de l'expérience* (voir Domjan, 1998, pp13-16 ; Pearce, 2008). Selon cette définition, le changement comportemental résultant de l'expérience passé n'a plus besoin d'être adaptatif pour être considéré comme de l'apprentissage.

D'une manière générale, la mémoire souffre moins d'une diversité de définition que le processus qui l'engendre, l'apprentissage. La mémoire est en effet largement considérée comme le stockage de l'information, subjectivement le souvenir. Plusieurs phénomènes sont souvent regroupés sous le terme de mémoire : la consolidation, la rétention, la récupération

et l'utilisation de l'information mémorisée. On définira en revanche, plusieurs types de mémoires, classés en fonction de divers facteurs tels les processus neurologiques impliqués et la résistance aux agents amnésiques. Classiquement, les travaux réalisés chez la drosophile ont permis de définir quatre types de mémoire (Fig. 1) : la **mémoire à court terme** (STM pour *Short-Term Memory*), la **mémoire à moyen terme** (*Intermediate-Term Memory* ou ITM), la **mémoire résistante à l'anesthésie** (*Anaesthesia-Resistant Memory* ou ARM) et enfin la **mémoire à long terme** (*Long-Term Memory* ou LTM). Contrairement à ce que l'on pourrait penser, la distinction entre ces différents types de mémoire ne repose pas entièrement sur la durée de persistance de la marque mnésique, c'est-à-dire de l'information mémorisée. En effet, le moment de la formation de chacun des types de mémoire, ainsi que les mécanismes impliqués dans leur formation, contribuent à les définir.

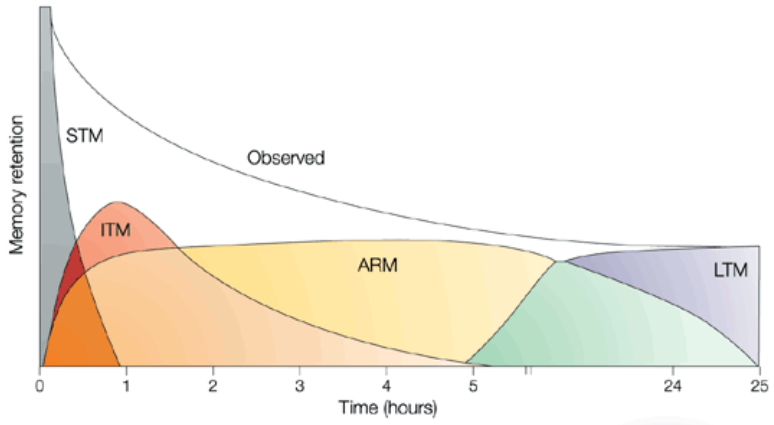

FIGURE 1 – Chronologie de la formation de la mémoire chez la drosophile. STM : mémoire à court terme ; ITM : mémoire à moyen terme ; ARM : mémoire résistante à l'anesthésie ; LTM : mémoire à long terme. D'après Tully *et al.*, 2003

Chez la drosophile par exemple, la formation de STM débute immédiatement après l'entrainement, c'est à dire après l'apprentissage. Cette forme de mémoire décline en revanche très rapidement, i.e. en l'espace de quelques minutes, de quelques secondes. Hebb (1949), définit la mémoire à court terme comme une réverbération continue de l'activité d'ensembles de cellules (ensembles de neurones interconnectés qui encodent un stimulus). Les neurones s'activent réciproquement pendant un certain temps, à la suite de la stimulation

originelle. Probablement due à la dégradation des neurotransmetteurs, cette réverbération cesse, établissant ainsi la fin de la mémoire à court terme. De manière empirique, la durée de la mémoire à court terme est parfois déterminée en fonction du temps nécessaire pour observer un effet des traitements amnésiques réalisés post-entrainement. Par exemple, si l'injection d'inhibiteurs de synthèse protéique provoque une perte de l'information mémorisée 8h après l'apprentissage mais n'a aucun effet 4h post-entrainement, la mémoire à court terme sera définie comme persistant au moins 4h mais pas plus de 8h.

La formation de la mémoire à moyen terme (ITM) débute elle quelques minutes après l'entrainement et peut persister pendant plusieurs heures. Sa formation requiert la synthèse de protéines mais n'est pas dépendante de la synthèse d'ARN.

La mémoire résistante à l'anesthésie (ARM) se forme dans l'heure qui suit l'entrainement mais peut persister plusieurs jours. Il s'agit d'une forme de mémoire consolidée, résistante aux perturbations des patterns d'activité du cerveau (i.e. de l'activité neuronale) et ne nécessitant pas la synthèse de protéines.

Enfin, l'établissement de la mémoire à long terme (LTM) requiert un entrainement répété et se met en place plusieurs heures après l'apprentissage et peut persister jusqu'à plusieurs semaines chez la drosophile (Tully *et al.*, 2003). Sa formation fait appel à la synthèse de protéines et d'ARN (Sutton *et al.*, 2002).

Chez la drosophile, il a été mis en évidence que, dans le contexte du conditionnement classique, plus précisément dans le cas de conditionnement olfactif aversif, la mémoire à long terme (LTM) ne se forme qu'à la suite de sessions de conditionnement répétées et séparées par des intervalles de repos (conditionnement espacé), tandis que la mémoire résistante à l'anesthésie (ARM) se formera également si des événements consécutifs de conditionnement se succèdent les uns après les autres (conditionnement massé) (Tully *et al.*, 1994). La mémoire à long terme est plus stable mais, contrairement à l'ARM, la synthèse de protéines est nécessaire à sa formation. Par conséquent, la formation de LTM est plus couteuse en énergie ainsi qu'en terme de fitness (Mery et Kawecki, 2005).

L'étude de ces différentes formes de mémoire chez des insectes tels que la drosophile, dont le génome est entièrement séquencé et cartographié, permet de "disséquer génétiquement" la formation de la mémoire. En utilisant des souches mutantes pour un gène en particulier, les effets de mutations uniques ont pu être analysés pour chaque type de mémoire, mettant ainsi en évidence une véritable "voie" de formation de la mémoire.

Par exemple, l'acquisition de l'association odeur-choc est interrompue chez les mutants *fasciclin II*, *latheo* et *linotte*. La formation de STM est interrompue chez les mutants *dunce*, *rutabaga* et *volado*, d'ITM chez les mutants *amnesiac* et *DC0*, l'ARM chez les mutants *radish* et la LTM chez les mutants *nalyot* notamment (Tully *et al.*, 2003). De plus, il est désormais connu que la voie génétique de formation de la mémoire passe par des étapes séquentielles, successive et également par des étapes se déroulant en parallèle. Par exemple, des interruptions de l'acquisition ou de la formation de STM ou d'ITM affectent également les étapes se situant en aval des processus impliqués (on parle également d'effet *downstream*). En revanche, la LTM n'est pas perturbée chez les mutant *radish* (dont l'ARM est interrompue) et l'ARM n'est pas perturbée chez les mutant *nalyot* (incapables de former la mémoire à long terme). Ces résultats indiquent des voies de formations fonctionnellement indépendantes pour ces deux types de mémoire (Fig. 2). Cette indépendance peut être expliquée par des voies biochimiques différentes, ou encore par des traitements dans différentes régions anatomiques du cerveau (Tully *et al.*, 2003).

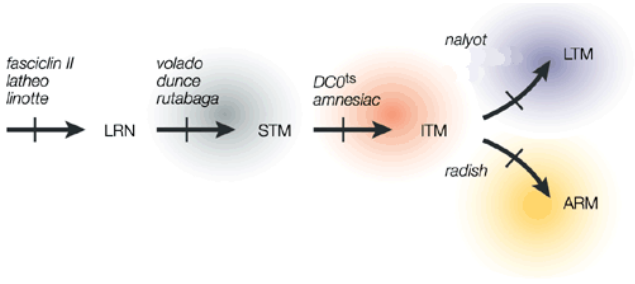

FIGURE 2 – Voie génétique de la formation de la mémoire chez la drosophile. La position des mutations indiquent le type de mémoire perturbé par ces dernières. D'après Tully *et al.*, 2003

Apprentissages non-associatifs et associatifs.

Classiquement, on distingue deux grands types d'apprentissage, selon qu'il soit associatif (c'est-à-dire correspondant à l'apprentissage de l'association entre différents stimuli) ou non-associatif.

Parmi les non-associatifs, l'**habituation** correspond à la diminution de la probabilité ou de l'amplitude d'une réponse inconditionnée, en fonction de la présentation répétée du stimulus déclenchant la réponse. Chez les insectes, ce phénomène a été observé à la fois au niveau comportemental et neural, i.e. sur des fibres nerveuses isolées. Bien que ce phénomène ait été décrit et définit à partir d'observation chez les vertébrés (Thompson et Spencer, 1966), on le retrouve chez de nombreux invertébrés, parmi lesquels des protozoaires, des planaires, des annélides, des échinodermes, des mollusques et des arthropodes (Corning *et al.*, 1973).

A l'opposé de l'habituation, la **sensibilisation** correspond à une augmentation de la probabilité ou de l'amplitude d'une réponse. La définition donnée par Razran (1971, p. 58) fait référence à l'augmentation, plus ou moins permanente, d'une réaction innée, suite à des stimulations répétées. Cette augmentation de la réactivité peut se manifester de deux façons : a) par l'augmentation de la fréquence et de l'intensité de la réponse ainsi que par une diminution de la latence et du seuil de réaction, et b) par pseudo-conditionnement ou par de nouvelles réponses à des stimuli originellement inadéquats et non appariés (Razran, 1971, p. 78).

L'exemple utilisé par Mikulas (1978) illustre parfaitement ce concept, par ailleurs souvent source de confusion (Corning *et al.*, 1973). Il s'agit de l'exemple d'un chien auquel on délivre un stimulus électrique à la patte arrière (Mikulas, 1978). Ces stimuli entrainent une réponse musculaire de la patte. Bien que l'intensité du stimulus reste constante au cours de l'expérience, suite à plusieurs stimulations répétés, la réponse du chien est plus prononcée (augmentation de l'intensité) et plus rapide (diminution de la latence). Cette augmentation de la réactivité correspond à la sensibilisation. De la même manière, après un certain

nombre de stimulations, le nombre de fois où l'animal répond augmente (augmentation de la fréquence) et l'intensité minimale nécessaire pour déclencher une réponse peut diminuer (diminution du seuil).

Le pseudo-conditionnement fait quand à lui référence au renforcement d'une réponse à un stimulus précédemment neutre, suite au déclenchement répété d'une réponse par un autre stimulus délivré indépendamment. En d'autres termes, le stimulus qui déclenche la réponse n'est pas apparié avec le stimulus neutre. Reprenons l'exemple du chien, proposé par Mikulas (1978), et considérons qu'entre les stimuli électriques (c'est-à-dire en évitant tout contingence entre les stimuli), un son est délivré à l'animal. Dans un premier temps, le son ne déclenche pas la réponse produite par le stimulus électrique. En revanche, après un certain nombre d'essais, c'est-à-dire de répétitions de la réponse produite par l'électrostimulation, on constate que l'animal répond désormais au son, par une contraction musculaire de la patte, bien que le son et le stimulus électrique n'aient jamais été appariés. Cette augmentation de la réponse de la patte au son, est un exemple de pseudo-conditionnement.

Parmi les formes d'apprentissages associatifs, le plus célèbre est probablement le **conditionnement classique**, décrit par Pavlov au début du XXème siècle. Il correspond à un changement comportemental observé en réponse à une association entre deux stimuli. Un stimulus inconditionnel (US pour *Unconditional Stimulus*), qui induit systématiquement une réponse inconditionnée (UR pour *Unconditioned Response*) chez l'animal testé, est apparié avec la présentation d'un stimulus conditionnel (CS pour *Conditional Stimulus*) qui lui, ne provoque aucune réponse. Suite à l'entrainement, c'est-à-dire à la répétition d'essais au cours desquels US et CS sont appariés, le CS devient alors un stimulus conditionné, déclenchant la réponse, désormais appelée réponse conditionnée (CR pour *Conditionned Response*).

Ce type de conditionnent requiert une contingence temporelle entre la présentation de l'US et du CS de telle façon que le CS soit informatif vis-à-vis de l'US, c'est-à-dire de façon à ce que l'animal puisse établir une relation entre la présentation des deux stimuli

(Rescorla, 1988).

Le conditionnement classique, ou Pavlovien, se distingue d'un autre type de conditionnement dit **opérant** ou **instrumental**. Il est ici fait référence à type de conditionnement associatif, dans lequel une contingence est établie entre la réponse d'un organisme et un renforcement. Cette situation correspond à celle décrite dans les expériences classiques de Skinner (1938), au cours desquelles il entrainait des rats et des pigeons à presser un levier, afin d'obtenir une récompense ("*SkinnerBox*"). Dans ces expériences, le sujet est souvent capable de réaliser une grande variété de "sorties" comportementales. L'expérimentateur choisi une sortie (*output*) adaptée, c'est-à-dire la réponse R (e.g. presser un levier), qui sera appariée avec la présentation d'un stimulus inconditionnel (US, par exemple de la nourriture en guise de récompense). Souvent, un stimulus discriminatif (SD, par exemple un signal lumineux) est présenté lorsque la contingence R-US est réalisée. Après une période d'entrainement, le sujet démontrera une augmentation de la réponse R - ce qui correspond à la réponse conditionnée (CR pour *Conditioned Response* ; ici presser le levier) - même en l'absence de l'US, si l'association R-US à été mémorisée. On dit alors que le sujet a appris au sujet d'une contingence remarquable entre son propre comportement et un élément de son environnement (i.e. "*between its own behavior and some part of the world*" ; Brembs, 1996). Les conséquences positives (renforcement positif, récompense) ou négatives (renforcement négatif, punition) d'un comportement entrainent donc, en retour, l'augmentation ou la diminution de la fréquence de cette réponse comportementale. Enfin, il est commun de regrouper sous le terme d'apprentissage complexe diverses formes d'apprentissages non-élémentaires, tels l'**apprentissage contextuel**, la **catégorisation** et l'**apprentissage de règles abstraites**.

Toutes ces formes d'apprentissage permettent aux individus d'établir des règles prédictives au sein d'un environnement souvent variable et complexe. Depuis le début du XX$^{\text{ème}}$ siècle, de nombreuses études expérimentales ont été menées afin de mettre en évidence et de caractériser les capacités d'apprentissage d'animaux allant des protozoaires aux humains et en leur faisant accomplir une grande variété de tâches.

Chez les insectes, la capacité d'apprendre et mémoriser des informations a également été mise en évidence. Dès 1957, Baxter démontrait la possibilité d'habituer le comportement de fuite de la blatte *Periplaneta americana* déclenché par un puff d'air. Des formes d'apprentissages associatifs ont été mises en évidence par Frings (1941) chez la mouche *Cynomyia cadaverina* et les premiers travaux réalisés chez l'abeille domestique concernant la perception des formes (e.g. von Frisch, 1914), des couleurs (e.g. von Frisch, 1914) et des odeurs (e.g. von Frisch, 1919) ont en commun une illustration implicite de conditionnement associatif.

Depuis ces travaux, le nombre d'espèces d'insectes chez lesquelles les capacités d'apprentissage ont été démontrées est en constante augmentation. Malgré cela, l'immense majorité des études sur le sujet ont été réalisées sur deux modèles : l'abeille domestique et la drosophile. En effet, une révision récente de la littérature révèle que parmi les publications consacrées à l'étude de l'apprentissage chez les insectes, 60 % d'entre elles ont la drosophile pour modèle d'étude (e.g. Dubnau et Tully, 1998 ; Xia *et al.*, 1998 ; Barron et Corbet, 1999 ; Mery et Kawecki, 2002 ; Siwicki et Ladewski, 2003...). L'abeille est utilisée dans 23 % des publications (e.g. Bitterman *et al.*, 1983 ; Bitterman, 1996 ; Menzel, 1999 ; Deisig *et al.*, 2001 ; Menzel et Giurfa, 2001 ; Giurfa, 2003 ; Carcaud *et al.*, 2009 ; Srinivasan, 2010...). Deux autres modèles d'étude ressortent également : les fourmis, qui représentent 10 % des publications consacrées à l'apprentissage chez les insectes, et les guêpes parasitoïdes, qui représentent 6 % des publications (e.g. Dukas et Duan, 2000 ; Wehner *et al.*, 2006). Quatre modèles sont donc l'objet de 99 % des études concernant l'apprentissage et la mémoire chez les insectes et deux d'entre eux, la drosophile et l'abeille domestiques sont aujourd'hui considérés comme des modèles classiques pour l'étude de ces processus (Source : *Web of Science*).

Ce que nous apprennent les modèles insectes dits classiques.

Les raisons du succès de l'abeille et de la drosophile en tant que modèle de prédilection pour l'étude de l'apprentissage, reposent sur plusieurs facteurs. Historiquement tout d'abord, ces insectes ont fait, depuis le début du XX$^{\text{ème}}$ siècle, l'objet de nombreuses études, centrées sur leur comportement.

La drosophile *Drosophila melanogaster* s'est révélée dans les années 1900 comme étant un modèle permettant d'accroître exponentiellement les connaissances en biologie du développement et en génétique. Cette augmentation du savoir culminant avec le prix Nobel d'Edward Lewis, Christiane Nüsslein-Volhard et Eric Wieschaus en 1995, qui leur fut décerné pour leurs découvertes concernant le contrôle génétique du développement embryonnaire.

En 1991, Martin Heisenberg et Reinhard Wolf publièrent une étude dans laquelle ils utilisèrent la drosophile, dans un simulateur de vol (Heisenberg et Wolf, 1991), afin d'analyser en détail le conditionnement opérant. Leur travail a ouvert de nombreux champs d'investigations concernant notamment la reconnaissance des patterns, les substrats biologiques de la mémoire et l'analyse de divers processus d'apprentissage (Brembs, 1996). Au-delà de l'aspect historique de ce modèle, la drosophile s'est également révélée être un modèle de référence pour l'analyse génétique (Pierce, 2004). Les caractéristiques biologiques de cet insecte présentent un grand nombre d'avantages, qui ont permis de réaliser des avancées rapides dans ce domaine. Entre autres, l'élevage est peu couteux et ne nécessite que peu d'espace et peu d'équipement ; le temps de génération est court (une dizaine de jours à température ambiante), ainsi plusieurs générations peuvent être étudiées en l'espace de quelques semaines ; sa fécondité est élevée (environ 100 œufs par jour et par femelle) ; la présence de chromosomes géants dans les glandes salivaires des larves matures ; la présence de seulement quatre paires de chromosomes, trois autosomes et un chromosome sexuel ; l'absence de recombinaison méiotique chez les mâles ; le génome séquencé dans son intégralité en 2000 (Adams *et al.*, 2000), etc.

Ainsi, la drosophile permet d'étudier les mécanismes génétiques fins sous-jacents à la formation de la mémoire, à la réalisation de certaines formes d'apprentissage et l'utilisation de mutants permet d'identifier les voies génétiques et moléculaires, responsables de la réalisation des comportements (Brembs, 1996 ; Tully *et al.*, 2003). La possibilité de travailler, rapidement, sur plusieurs générations de mouche a également permis d'étudier l'évolution des capacités d'apprentissage au fil des générations (Mery, 2006) et d'établir des liens entre apprentissage, évolution et spéciation (Mery et Kawecki, 2002).

Chez l'abeille, les premiers travaux d'importance sont ceux de Karl von Frisch (1914) qui conduiront à une connaissance détaillée de la richesse comportementale de cet insecte (von Frisch, 1967). La complexité des comportements réalisés par l'abeille, a justifié son emploi dans les études sur les capacités cognitives des insectes (Menzel et Giurfa, 2001). Les abeilles sont en effet capables de naviguer efficacement sur plusieurs kilomètres entre leur ruche et les patchs de fleurs, sources de nourriture, et elles sont dotées de capacités sensorielles et de performances motrices très développées. Ces caractéristiques ont su être exploitées dans un contexte expérimental, pour l'étude de leurs capacités cognitive (Menzel et Giurfa, 2001). Parmi les paradigmes les plus fréquemment utilisés, celui du conditionnement olfactif de la *réponse d'extension du proboscis* (ou PER), permet d'entrainer des abeilles immobilisées à répondre à des stimuli olfactifs (Takeda, 1961 ; Bittermann *et al.*, 1983). Il s'agit de toucher les antennes d'une abeille affamée avec une goutte de solution sucrée, ce qui provoque la PER de l'abeille qui cherche à atteindre la solution sucrée. L'odeur neutre (CS) (c'est-à-dire ne déclenchant pas la PER chez les animaux naïfs) est appariée avec la présentation de la solution sucrée (US). Après une période d'entrainement, l'abeille conditionnée répond à l'odeur et étirant son proboscis. Ce paradigme expérimental a permis d'étudier les bases physiologiques de l'apprentissage olfactif. En effet, couplé à des observations neuro-anatomiques et à des techniques d'imagerie calcique, il a été possible d'établir des liens entre l'activité des structures du cerveau de l'abeille et ses capacités d'apprentissage (Hammer, 1993 ; Mauelshagen, 1993 ; Galizia et Menzel, 2000 ; Menzel et Giurfa, 2001 ; Malun *et al.*, 2002 ; Hourcade *et al.*, 2009, 2010). D'autres aspects des ca-

pacités cognitives ont peut être abordés en ayant recours au conditionnement du vol de rapprochement des abeilles à un stimulus visuel récompensé (von Frisch, 1967) ou encore grâce à l'utilisation de labyrinthe en Y (Giurfa *et al.*, 1996). Chez les abeilles, l'apprentissage n'est plus uniquement un objet d'étude *per se* mais est devenu un outil permettant de répondre à des questions concernant par exemple la capacité à discriminer des stimuli proches et à déterminer les différences minimales perceptibles par l'insecte (Giurfa *et al.*, 2001).

En près de cent ans d'expériences, la drosophile et l'abeille se sont donc imposées comme modèles pour l'étude des capacités d'apprentissage des insectes, permettant la mise en place de paradigmes expérimentaux de référence et la caractérisation des mécanismes génétiques et neurobiologiques sous-jacents. Leur succès en tant que modèles classiques n'est pas dû à la présence de capacités cognitives a priori plus développées que chez d'autres insectes (blattes, fourmis, guêpes et papillons ont également révélés des capacités d'apprentissage ; Daly et Smith, 2000 ; Dukas et Duan, 2000 ; Seidl et Wehner, 2006 ; Decker *et al.*, 2007). En revanche, leur longue histoire en tant que modèles d'étude du comportement et la connaissance approfondie de leur biologie ont permis de placer ces insectes dans des contextes expérimentaux permettant de mettre en évidence et de caractériser leur capacités cognitives.

Bien que les pressions de sélection ayant conduit à l'évolution de telles capacités d'apprentissage soient importantes chez les modèles classiques et chez l'abeille en particulier (pressions de sélections liées notamment à la vie en société), chez les insectes hématophages, des pressions de sélection extrêmement fortes s'exercent également. Ces pressions sont notamment liées au régime alimentaire particulier, qui place par exemple l'insecte dans une situation où sa source de nourriture (le sang d'un hôte) ou plus exactement sa proie (l'hôte) joue aussi le rôle de prédateur (via son comportement défensif et antiparasitaire, ou encore par prédation directe). On s'attend donc à observer des capacités d'apprentissages développées chez les insectes se nourrissant de sang, et dont un certain nombre sont vecteurs de maladies animales et humaines.

Cependant l'idée que les vecteurs soient capables d'apprendre au travers de leur expérience, a été laissée de côté pendant longtemps (Clements, 1999), en dépit de l'accumulation d'indices, principalement chez les moustiques, de l'existence d'une mémoire spatiale et de l'implication de l'apprentissage dans la localisation des hôtes et le choix des hôtes (voir McCall et Kelly, 2002 pour plus de références).

Le paradoxe hématophage

Pourtant, il existe un large consensus concernant l'apprentissage et la mémoire chez les insectes hématophages. Il serait en effet surprenant pour de nombreux auteurs que ces insectes ne soient pas capables d'apprentissage, notamment d'apprentissage spatial. En effet, bien que l'emplacement des sources de nourriture, des partenaires sexuels et des sites d'oviposition puisse varier sur de longues échelles de temps (i.e. ne serait-ce que sur quelques années), l'emplacement de certaines ressources va rester constant à l'échelle d'un cycle gonotrophique ou le long de la vie d'un insecte. Par conséquent, le bénéfice, qu'apporterait la capacité d'apprentissage, et notamment d'apprentissage spatial, parait évident.

L'idée que les hématophages puissent être doués de mémoire a été évoquée, pour la première fois en 1934 chez *Anopheles maculipennis*, pour expliquer la fidélité des moustiques au site d'alimentation (Collado et Castellá, 1934). Il s'agit en réalité d'un groupe de six espèces de moustiques, dont certaines sont impliquées dans la transmission du paludisme. Bien que ces espèces soient morphologiquement indissociables, un isolement reproductif les sépare. Potentiellement, plusieurs espèces de moustiques pouvaient donc être capables de mémoriser des informations relatives à leur environnement. De même, d'autres espèces de moustiques retournent essaimer sur le même site lors d'occasions successives (voir McCall et Eaton (2001) et les références incluses). Cependant, dans toutes ces études, l'implication de l'apprentissage et de la mémoire dans ces phénomènes, n'a pas été clairement démontrée, notamment parce que d'autres explications alternatives n'avaient pas pu être écartées.

L'étude de Charlwood *et al.* (1988) menée en Papouasie-Nouvelle-Guinée, est probablement la première à s'être focalisée explicitement sur la mémoire chez un insecte vecteur. Les auteurs sont parvenus à mettre en évidence le fait que les femelles moustiques de l'espèce *Anopheles farauti*, relâchées loin du village où elles s'étaient précédemment nourries, se dispersent d'avantage que les femelles relâchées localement. Après une alimentation à réplétion et les œufs maturés, les moustiques vont répondre aux signaux émanant des sites d'oviposition (Klowden et Blackmer, 1987). Pour les auteurs, la mémoire spatiale d'un environnement connu, a permis aux femelles de repérer plus facilement et plus rapidement des sites d'oviposition, sans recourir à de longs vols exploratoires. Un comportement similaire à été observé chez les phlébotomes (Kelly et Dye, 1997 ; Campbell-Lendrum *et al.*, 1999). Cependant, dans ces travaux le manque de contrôle des conditions expérimentales ne permet pas d'écarter d'autres hypothèses pouvant expliquer ces comportements.

En 1991 l'implication de l'apprentissage et de la mémoire est cette fois-ci évoquée concernant le choix des hôtes (Hii *et al.*, 1991). Ces résultats seront renforcés par les travaux de Mwandawiro *et al.* (2000), qui suggèrent que le taux d'alimentation du moustique *Culex vishnui* est supérieur lorsque les individus s'alimentent sur un hôte sur lequel un précédent repas sanguin a été réalisé. Des résultats similaires ont été obtenus chez la mouche Tsé-tsé, *Glossina palpalis gambiensis*. Plus précisément, le choix de l'hôte lors de repas subséquents, semble être influencé par l'hôte sur lequel le dernier repas sanguin a été obtenu (Bouyer *et al.*, 2007). Une fois de plus, l'apprentissage et la mémoire sont maintenus à l'état d'hypothèses étant donné que d'autres alternatives ne sont pas écartées (e.g. adaptation physiologique à un type de sang particulier, différentes facilités d'ingestion de sangs de viscosités différentes, différents comportements antiparasitaires, etc.).

La valeur adaptative de telles capacités dans le contexte de la recherche d'hôtes ne peut être qu'élevée. En effet, il existe de grandes différences entre les hôtes concernant leurs comportements défensifs, que cela soit au niveau inter ou intra-spécifique. Du point de vue des insectes, être capable d'apprendre et de mémoriser des associations entre les signaux émanant des hôtes d'une part et leur comportement défensif d'autre part, leur

permettrait de maximiser leur succès d'alimentation ainsi que de minimiser le risque de se faire blesser ou tuer au cours de l'opération. En d'autres termes, cela leur permettrait de s'alimenter préférentiellement sur les hôtes les moins défensifs (Kelly, 2001), en apprenant à éviter les hôtes les plus défensifs dans les situations où des hôtes alternatifs peuvent être facilement trouvés.

Depuis ces travaux, l'effort de recherche s'est intensifié et d'autres résultats suggérant l'implication de l'apprentissage et de la mémoire, ont été obtenus. Ainsi, chez les moustiques, le choix des sites d'oviposition (McCall et Eaton, 2001), la fidélité au site (McCall *et al.*, 2001) ainsi que la réponse aux insecticides et aux répulsifs (Kaur *et al.*, 2003) sembleraient être influencés par les capacités cognitives des insectes. Cependant, ces travaux étant réalisés dans des conditions naturelles ou partiellement contrôlées, ils n'apportent pas de démonstration claire de l'existence des capacités d'apprentissage. Plus récemment, des procédures de conditionnement olfactif ont été employées chez le moustique *Culex quinquefasciatus* (Tomberlin *et al.*, 2006), apportant ainsi un contrôle plus important du contexte expérimental.

Comme l'ont précédemment souligné Alonso et Schuck-Paim (2006), améliorer la compréhension des capacités d'apprentissage des vecteurs de maladies, pourrait permettre de comprendre dans quelle mesure l'expérience individuelle des insectes influence le choix des hôtes ainsi que la capacité des vecteurs à transmettre les maladies. En particulier, nous ne savons pas dans quelle mesure la recherche des hôtes par les hématophages repose sur des comportements innés, l'expérience individuelle ou une combinaison des deux.

Pourtant, un grand nombre d'auteurs s'accordent à dire que les investigations sur les capacités cognitives des vecteurs de maladies pourraient améliorer la compréhension des mécanismes sous-jacents à la préférence d'hôte (e.g. pourquoi certaines espèces d'hôtes sont préférées à d'autres), et expliquant la distribution hétérogène des vecteurs au sein des populations d'hôtes (e.g. pourquoi certaines personnes sont préférées à d'autres) (Hasibeder et Dye, 1988 ; Kelly et Thompson, 2000 ; Kelly, 2001 ; McCall et Kelly, 2002). Or, la distribution spatiale d'une population d'insectes hématophages au sein des hôtes vertébrés

ainsi que l'hétérogénéité dans le comportement de piqûre ont toutes deux des conséquences épidémiologiques dans la transmission de maladies importantes, telles que le paludisme, la leishmaniose ou encore la maladie de Chagas (Dye et Hasibeder, 1986 ; Hasibeder et Dye, 1988). Woolhouse *et al.* (1997) ont estimé qu'en effet, environ 20 % d'une population d'hôtes contribuent à environ 80 % du potentiel net de transmission.

Parmi les insectes hématophages, les triatomines, vecteurs de la maladie de Chagas (ou Trypanosomiase américaine), ont retenues toute notre attention. En effet, ces insectes présentent des similitudes avec l'abeille et la drosophile qui constituent des avantages, en comparaison d'autres insectes vecteurs, pour l'étude de l'apprentissage et la mémoire.

Tout d'abord, d'un point de vue pratique, la taille et la morphologie de ces insectes permettent d'adapter des paradigmes expérimentaux largement utilisés et validés chez les modèles classiques. La manipulation et la contention de ces insectes est possible et mentionnée dans de nombreux travaux les concernant (e.g. Barrozo et Lazzari, 2004a, b). Qui plus est, ces insectes sont considérés comme des modèles classiques pour l'étude de la physiologie, depuis les travaux de W.B. Wigglesworth dans les années 1930.

Cet insecte hématophage vit en forte association avec ses hôtes vertébrés (voir annexe). Pendant la journée, les punaises restent abritées, en akinésie, dans leurs refuges (nids d'oiseaux, terriers de rongeurs, habitations humaines...), qui leur procurent des conditions environnementales stables (e.g. peu de variations de température et d'humidité), ainsi qu'une protection contre les prédateurs. Les punaises sortent de leur akinésie [1] en début de nuit, sous le contrôle d'une horloge circadienne (Lazzari, 1992), et vont répondre aux signaux émis par leurs hôtes (i.e. des composés volatiles émis par la respiration ou à travers la peau) et portés par des courants d'air. Arrivés à proximité d'un hôte, les punaises utilisent des informations thermiques pour localiser les vaisseaux sanguins dans lesquels elles pourront prélever leur aliment (Ferreira *et al.*, 2007). De plus, la chaleur est le seul facteur à la fois nécessaire et suffisant pour induire une extension du proboscis (PER). En fin de nuit, les punaises vont répondre aux phéromones d'agrégation déposées à l'entrée des

1. c'est à dire une réduction de toute forme d'activité motrice avec une tendance à l'immobilitée

refuges, sous l'influence directe de l'augmentation de l'intensité lumineuse (Bodin *et al.*, 2008), et vont s'agréger les unes aux autres par thigmotactisme [2], à l'intérieur des refuges (Lorenzo Figueiras et Lazzari, 1998b). Il est à noter que les réponses des punaises aux signaux des hôtes sont modulées par l'état nutritif et physiologique des insectes (Bodin *et al.*, 2009a, b), il est donc de la plus grande importance de tenir compte de ces facteurs lorsque l'on étudie la réponse comportementale de ces punaises.

L'accumulation de toutes ces connaissances concernant leur biologie, permet de contrôler les facteurs exogènes et endogènes qui pourraient perturber la mise en place d'expériences de conditionnement. On sait par exemple, que ce sont des insectes hémimétaboles, hématophages obligatoires. En d'autres termes, d'une part les larves ressemblent à l'adulte et il est donc possible de s'affranchir des effets de la reproduction en travaillant avec des larves et d'autre part, il est possible de contrôler la motivation des insectes à répondre aux signaux de l'hôte. En revanche, les moustiques, qui sont également vecteurs de nombreuses maladies, ne présentent pas ces avantages. Les larves étant aquatiques et carnassières et les mâles adultes étant nectarivores, le travail sur les adultes implique de bien contrôler le cycle gonotrophique [3] des femelles, puisque le développement d'ovocytes induit une inhibition de la réponse aux signaux de l'hôte (Klowden et Briegel, 1994). De plus, chez certaines espèces de moustiques, l'alimentation sanguine des femelles est destinée à permettre le développement des ovocytes et la maturation des œufs, alors que l'activité métabolique est elle soutenue par une alimentation sucrée (nectar floral) (Klowden, 1997). Il est donc plus délicat d'obtenir un contrôle précis de l'état motivationnel des individus.

D'une manière plus générale, la réponse de ces insectes aux signaux de leurs hôtes a été largement caractérisée (Barrozo et Lazzari, 2004a,b ; Guerenstein et Lazzari, 2009 ; etc.) ainsi que la modulation de ces réponses par des facteurs endogènes (Bodin *et al.*, 2008, 2009a,b). L'utilisation des triatomines comme modèle permet ainsi de contrôler le contexte expérimental et l'état motivationnel des insectes.

2. ou tactisme de contact, c'est la tendance à suivre les discontinuités linéaires du milieu, c'est-à-dire à suivre les hétérogénéités rencontrées au cours du déplacement (obstacles, congénères...)

3. Ce cycle correspond à la succession des phénomènes physiologiques qui se produisent chez un insecte hématophages, entre le repas sanguin et la ponte des œufs.

Le séquençage du génome de la punaise hématophage *Rhodnius prolixus* (Triatominae, Reduviidae) étant en cours, pouvoir adapter des procédures de conditionnement développées chez l'abeille ou la drosophile, permettrait de bénéficier des connaissances accumulées chez ces insectes sur le contrôle génétique des capacités d'apprentissage.

L'objectif de notre travail n'est pas simplement de valider chez un autre insecte les résultats obtenus chez les modèles classiques, mais de fournir les outils qui permettront de répondre à d'autres questions, propres au modèle. Par exemple, la possibilité d'étudier la thermoperception, une modalité sensorielle propre à quelques espèces d'insectes seulement. Mais également, la possibilité de tester l'influence des parasites sur les capacités cognitives des insectes, et d'évaluer les capacités d'apprentissage chez un insecte au mode de vie hématophage.

Il s'agit donc ici d'exploiter le savoir accumulé concernant la biologie et la physiologie de ces insectes et de mettre en place des paradigmes d'apprentissage qui soient efficaces et reproductibles, afin d'évaluer et de caractériser les capacités cognitives des insectes vecteurs.

Une série de problématiques spécifiques sont abordées dans ce travail. La première concerne l'influence de l'apprentissage dans la reconnaissance et l'interprétation des signaux de l'hôte par les insectes (Partie 1). Plus précisément, nous avons cherché à savoir si les punaises sont capables d'associer un composé volatile (l'acide-L-lactique) émis par les hôtes, avec la possibilité d'obtenir un repas sanguin. Dans un second temps nous avons testé la capacité des punaises à associer la même odeur à la possibilité d'être soumises à une perturbation mécanique mimant le comportement défensif d'un hôte. L'objectif était ici de déterminer expérimentalement si la réponse des insectes aux signaux de l'hôte pouvait être modifiée par l'expérience individuelle. Nous avons ensuite replacé ces résultats expérimentaux dans un contexte plus naturel en évaluant l'influence de l'expérience des individus sur le choix de l'hôte. Dans ce cas, ce n'est plus un composé volatile, mais l'ensemble du bouquet d'odeurs d'hôtes vivants, qui ont été appariés avec la présentation d'une perturbation mécanique.

La seconde problématique abordée concerne la mise en place d'un paradigme expéri-

mental simple, efficace et reproductible permettant de caractériser les capacités d'apprentissage et de mémoire d'insectes hématophages (Partie 2). Nous avons pour cela adapté une procédure de conditionnement de la réponse d'extension du proboscis, développée chez l'abeille et la drosophile. Cette approche expérimentale nous a permis d'estimer la durée de rétention maximale de la marque mnésique, i.e. de l'information mémorisée.

Dans un dernier temps, nous avons cherché à identifier les facteurs endogènes susceptibles de moduler les capacités d'apprentissage des punaises hématophages (Partie 3). Plus précisément, nous nous sommes intéressés à la modulation journalière de ces dernières et avons testé l'implication d'horloges endogènes.

L'intégration de ces résultats permettra de mieux comprendre l'importance de l'apprentissage et de la mémoire chez les insectes vecteurs de maladies. L'ensemble des résultats sera discuté en intégrant l'impact de ces capacités cognitives sur la capacité vectorielle des insectes ainsi que sur les stratégies de contrôle employées, mais également en revenant sur les contraintes associées à l'hématophagie pour l'étude de l'apprentissage chez les insectes vecteurs.

Première partie

Conditionnement olfactif chez un insecte hématophage, *Rhodnius prolixus*

I. Apprentissage olfactif appétitif

Pour repérer et localiser leurs hôtes, les insectes hématophages détectent et suivent les signaux qu'ils émettent. A distance, la recherche d'hôtes passe par des signaux olfactifs émis notamment par la respiration et la transpiration (Lehane, 2005). Parmi ces signaux, l'importance du CO_2 et d'acides gras à courte chaîne a été soulignée par un certain nombre de travaux (Barrozo *et al.*, 2003 ; Barrozo et Lazzari, 2004a, b ;.Barrozo et Lazzari, 2006). Être capable d'apprendre et d'associer l'odeur d'un hôte sur lequel l'alimentation a pu être réalisée avec succès par le passé, serait avantageux pour l'insecte. En effet, pour les insectes hématophages, l'hôte joue à la fois de rôle de proie (source du repas sanguin) et celui de prédateur (via son comportement défensif et antiparasitaire). Pouvoir associer des composés volatiles à la possibilité d'obtenir un repas sanguin, permettrait aux insectes de repérer, à une distance à laquelle l'hôte n'est pas encore une menace, les hôtes dont la qualité nutritive du sang est riche et sur lesquels il est facile de s'alimenter.

L'étude qui suit ne constitue pas la première tentative visant à adapter une procédure de conditionnement olfactif appétitif chez *R. prolixus*. Dans leurs travaux Abramson *et al.* (2005) et Aldana *et al.* (2008) ont apparié la présentation d'un stimulus olfactif (CS) avec la possibilité pour l'animal de piquer à travers la membrane d'un alimentateur artificiel, source de chaleur (US). Cependant, les résultats obtenus par ces auteurs n'ont pas permis de démontrer l'existence de processus d'apprentissage. Une analyse détaillée des procédures utilisées révèle un manque de contrôle des paramètres expérimentaux (absence de véritable renforcement, nature des stimuli employés, etc.). En utilisant les connaissances accumulées

sur la biologie et la physiologie de ces insectes ainsi qu'en tenant compte du cadre théorique rigoureux qu'impose l'étude de l'apprentissage, nous avons pu véritablement adapter une procédure de conditionnement olfactif appétitif à la punaise *R. prolixus*.

Les résultats obtenus montrent que ces insectes sont capables d'associer une odeur de l'hôte isolée (l'acide L-lactique) avec la possibilité d'obtenir un repas sanguin. Alors que cette odeur, perçue par les insectes (Barrozo et Lazzari, 2004b), n'induit pas de réponse comportementale orientée (ni attraction, ni répulsion) chez des individus naïfs testés dans un olfactomètre, on observe une modification de la réponse à cette odeur (de l'indifférence à l'attraction) chez les individus entrainés. Une série de groupes contrôles a permis d'écarter l'hypothèse que l'effet observé soit dû à l'action d'un stimulus (CS ou US) agissant seul ou à l'action cumulée de la présentation des stimuli en l'absence de contingence (CS-US non-appariés).

Ces résultats représentent la première preuve de conditionnement olfactif chez les triatomines, vecteurs de la maladie de Chagas, et une des rares démonstrations disponibles chez les insectes hématophages en général.

Article publié : Vinauger, C., Buratti, L. & Lazzari, C. R. (2011a). *Journal of Experimental Biology* **214** : 3032-3038.

Chapitre 1

Learning the way to blood: first evidence of dual olfactory conditioning in a blood-sucking insect, *Rhodnius prolixus*. Part I: appetitive learning

Abstract. It has been largely assumed that the individual experience of insects that are disease vectors might not only contribute to animal fitness, but also have an important influence on parasite transmission. Nevertheless, despite the invested efforts in testing the capacity to learn and remember information in blood-sucking insects, only little conclusive information has been obtained to date. Adapting a classical conditioning approach to our haematophagous model, we trained larvae of *Rhodnius prolixus* to associate L−lactic acid, an odour perceived by these bugs but behaviourally neutral when presented alone, with food (i.e. positive reinforcement). Naive bugs − those exposed either to a conditioned stimulus (CS, L−lactic acid), unconditioned stimulus (US, heat) and reward (blood) alone or CS, US and reward in the absence of contingency − remained indifferent to the presence of an air stream loaded with L-lactic acid when tested in an olfactometer (random orientation), whereas the groups previously exposed to the contingency CS−US−reward (blood) were significantly attracted by L−lactic acid. In a companion paper, the opposite, i.e. repellence, was induced in bugs exposed to the contingency of the same odour with a negative reinforcement. This constitutes the first evidence of olfactory conditioning in triatomine bugs, vectors of Chagas disease, and one of the few substantiations available to date of olfactory conditioning in haematophagous insects.

Key words: associative learning, Chagas disease, host seeking, Triatominae, haematophagous.

1.1 Introduction

To locate and assess the quality of resources, insects rely on innate behaviours that are fine-tuned by individual experience (Dukas, 2008; Raine and Chittka, 2008). Learning and memory, understood as the insects' behavioural adjustments based on previous experience, are two mechanisms that could help them to integrate and adapt to local variations in their environment (Dukas and Bernays, 2000; Menzel, 2001). These mechanisms and, more precisely, associative learning, have been particularly well studied in bees and fruit flies, which can be considered as classical models (Siwicki and Ladewski, 2003; Srinivasan, 2010), and have also been described in other insects such as cockroaches, caterpillars and hymenopteran parasitoids (Alloway, 1972; Papaj and Lewis, 1993; Horridge, 1997; Wackers and Lewis, 1999; Lucchetta *et al.*, 2008; Costa *et al.*, 2010). These studies have provided large amounts of information on the genetic and neurobiological bases of learning as well as on the complexity of insects' cognitive abilities (Bitterman *et al.*, 1983; Bitterman, 1996; Dubnau and Tully, 1998; Xia *et al.*, 1998; Menzel, 1999; Menzel *et al.*, 2007; Menzel and Giurfa, 2001; Giurfa, 2003; Carcaud *et al.*, 2009).

There is, however, a remarkable lack of information on the cognitive abilities of blood-sucking insects and how learning and memory could affect the transmission of parasites. In particular, we do not know to what extent host seeking by blood-feeding insects relies on innate behaviour, individual experience or a combination of both. Yet, a number of authors agree that investigations about the cognitive abilities of disease vectors could improve our understanding of the mechanisms underlying host preference (e.g. why some people are bitten more than others), as well as the heterogeneous distribution of vectors amongst host populations (Hasibeder and Dye, 1988; Kelly and Thompson, 2000; McCall and Kelly, 2002). Both the spatial distribution of a population of blood-sucking insects amongst their vertebrate hosts as well as the heterogeneity in their biting behaviour have important epidemiological consequences for the transmission of important diseases, such as malaria, leishmaniasis or Chagas disease (Dye and Hasibeder, 1986; Hasibeder and Dye, 1988).

To date, most studies examining associative learning in medically important arthropods have focused on mosquitoes. The available evidence is related to site fidelity, oviposition preference, host preference, insecticide avoidance and sugar feeding (McCall *et al.*, 2001; McCall and Eaton, 2001; Alonso *et al.*, 2003; Kaur *et al.*, 2003; Jhumur *et al.*, 2006). Nevertheless, since most studies have been conducted in natural or not completely controlled conditions, few provide unquestionable evidence of learning in this group of insects (Alonso *et al.*, 2003; Alonso and Schuck-Paim, 2006). In haematophagous bugs, vectors of Chagas disease, some experimental studies have been conducted in order to investigate olfactory learning in these insects, but no evidence could be obtained (Abramson *et al.*, 2005; Aldana *et al.*, 2008).

From an evolutionary point of view, learning to recognize and remember the best hosts (e.g. less defensive and easiest to feed on) would represent an advantage. The information acquired during former foraging episodes could indicate what to avoid, as well as what to seek, during the subsequent episodes (McCall and Kelly, 2002). Olfaction plays a major role in host-seeking behaviour in haematophagous insects (Lazzari, 2009; Guerenstein and Lazzari, 2009). It allows insects to locate and recognize their host from a secure distance and to set up adaptive responses of approach or avoidance. In this context, host odours might represent a powerful and reliable source of information, perceived at a certain distance from the host, when the latter is not yet a potential predator for the insects. It has been recently shown that the same odour may either attract or repel haematophagous insects according to their physiological state (Bodin *et al.*, 2009a), but it remains unknown whether olfactory experience may also switch the behavioural response to a given odour.

Even though several olfactory conditioning procedures have been well standardized in some insect species, their application to haematophagous bugs is relatively difficult, because of the behavioural and physiological constraints associated with haematophagy (i.e. piercing mouthparts, heat-induced proboscis extension response and biting). In contrast to other fluid-feeding insects, such as bees, fruit flies or ants, blood-sucking insects need to pierce the skin (or a succedaneum) to obtain their food. So, to reward them in a controlled

way, haematophagous insects first need to be induced to bite (i.e. indispensable thermal stimulation) and then allowed to feed on a controlled volume of blood in order to keep their motivation high to feed throughout trials.

Here we attempt to shed some light on the learning abilities of blood-sucking insects, in particular on the way individual olfactory experience can influence further host-seeking behaviour. For this, we used the triatomine bug *Rhodnius prolixus*, a vector of the human-transmitted Chagas disease, as an experimental model and tested whether larvae are able to associate a neutral odour with the perspective of obtaining a blood meal. This species was chosen because: (1) its host-seeking behaviour has been intensively studied and the roles of odours and other cues in the context of host seeking have been analysed in detail, and (2) it constitutes a classical model in insect physiology since the seminal work of V. B. Wigglesworth and followers in the 1930s. We thus used the accumulated knowledge on *R. prolixus* biology in order to adapt classical conditioning procedures, developed in bees and other non-haematophagous models, to this blood-feeding model. For this, we developed an experimental paradigm allowing us to pair a behaviourally neutral (but perceived) olfactory stimulus with a blood reward. In a second paper, we used the same neutral stimulus, but in an aversive conditioning approach (Vinauger *et al.*, 2011b).

1.2 Material and Methods

1.2.1 Insects

As experimental subjects, we used fifth-instar larvae of *Rhodnius prolixus* Stål 1859 (family Reduviidae, subfamily Triatominae) in order to exclude any interference related to reproductive behaviour. Bugs were reared in the laboratory under a 12:12h light:dark illumination regime, at 26°C and 60-70 % relative humidity. Bugs were fed weekly with sheep heparinised blood, using an artificial feeder (Nunez and Lazzari, 1990). Fifth-instar larvae that had just moulted were isolated in individual plastic containers and starved until being tested, 15 days after their ecdysis, a necessary starvation time to ensure a high

motivation to feed (Bodin *et al.*, 2009b). It has previously been shown that these bugs hatch at the end of the night (Ampleford and Steel, 1982). Thus, for our experiments, bugs were collected in the morning following ecdysis. They were recognisable by their characteristic pale-pink colour.

All of the assays were conducted in a room maintained at 25± 2℃, 40-60 % relative humidity. The experiments were carried out during the first hours of the scotophase (i.e. the dark period, or night-time, of the light cycle) because triatomines display a peak of activity throughout this period, which corresponds to the moment bugs leave their refuges to seek for host-emitted cues and exhibit the highest sensitivity to host odours (Lazzari, 1992; Barrozo *et al.*, 2004; Bodin *et al.*, 2008).

1.2.2 Conditioning procedure

Experiments were led using L-(+)-lactic acid (LA) as conditioned stimulus (CS). This volatile is emitted by vertebrates and has been detected on human skin (Acree *et al.*, 1968; Bernier *et al.*, 2000). LA was demonstrated to be the major component of perspiration (Braks *et al.*, 1999), excreted at concentrations between 0.5 and 5 $mg.ml^{-1}$ (Eiras and Jepson, 1991; Cork and Park, 1996; Geier *et al.*, 1996). In triatomine bugs, it is behaviourally neutral when presented alone, but lowers the response threshold of the bugs to CO_2 and fatty acids when combined with them (Barrozo and Lazzari, 2004a; Barrozo and Lazzari, 2004b). Thus, it is perceived by bugs, but no oriented response is induced, representing a good controlled stimulus for testing olfactory learning.

Training procedure: the artificial feeder. To investigate the ability of triatomines to associate an odour with a positive reinforcement, we set up a device allowing us to pair the presentation of an odorant (LA, CS) with a blood reward (appetitive conditioning), using heat as unconditioned stimulus (US) to evoke attraction and biting (unconditioned response) for recovering blood (positive reinforcement).

The device consisted of an artificial feeder (Fig. 1.1) allowing us to offer controlled

quantities of blood and to expose 10 bugs at a time to the CS and/or the US, along with the positive reinforcement. It was composed of ten 250µl micropipette cones whose tips were cut and sealed with Parafilm® through which bugs were able to bite (2 mm tip diameter, 3 cm height). Each tube was filled, for each trial, with 15µl of sheep heparinised blood and placed in a taped aluminium block (48 x 13 x 0.4 cm) equipped with a flat electric resistance. A thermostat kept the temperature of the blood at 33°C, which roughly corresponds to a host body surface temperature. The aluminium block was isolated with a polystyrene foam plate (48 x 13 x 0.5 cm) that was pierced to make the tubes accessible, in order to present the thermal stimulus only through the tube membrane. The bugs were individually placed in plastic containers (11.7 cm height and 1.5 cm diameter), the tops of which were covered with a fabric mesh, allowing the insects to access the feeding tubes. A piece of filter paper inside each container allowed the bugs to climb up and reach the feeder. An individual air-delivery system was connected to each container, which could carry the CS (or not) (Fig. 1.2).

Air currents were generated by an air pump, and flow rate was regulated by a flow meter equipped with a needle valve. The airflow was split in two circuits. Each circuit was made of silicone tubing conducting the air current through a glass bottle containing a piece of filter paper (2.5 cm^2). One of them was soaked with 50 µl of distilled water and the other with 50 µl of LA solution (100 µg LA 50 μl^{-1} distilled water). The choice of the circuit was controlled by a solenoid valve; this enabled us to subject the bugs to streams of either clean ambient air or air loaded with LA at the same temperature, flow rate and relative humidity by activating the solenoid valve. Furthermore, it allowed a precise control of the stimulus duration.

Before the training session began, and before each trial, bugs were allowed to familiarize themselves for 2 min inside the plastic container in the absence of stimulation, except for the delivery of a clean air current. After this time, the air current loaded with LA was delivered for 1 min. The artificial feeder was then placed over the containers and the LA stimulation was maintained for one further minute. From this moment, bugs were allowed

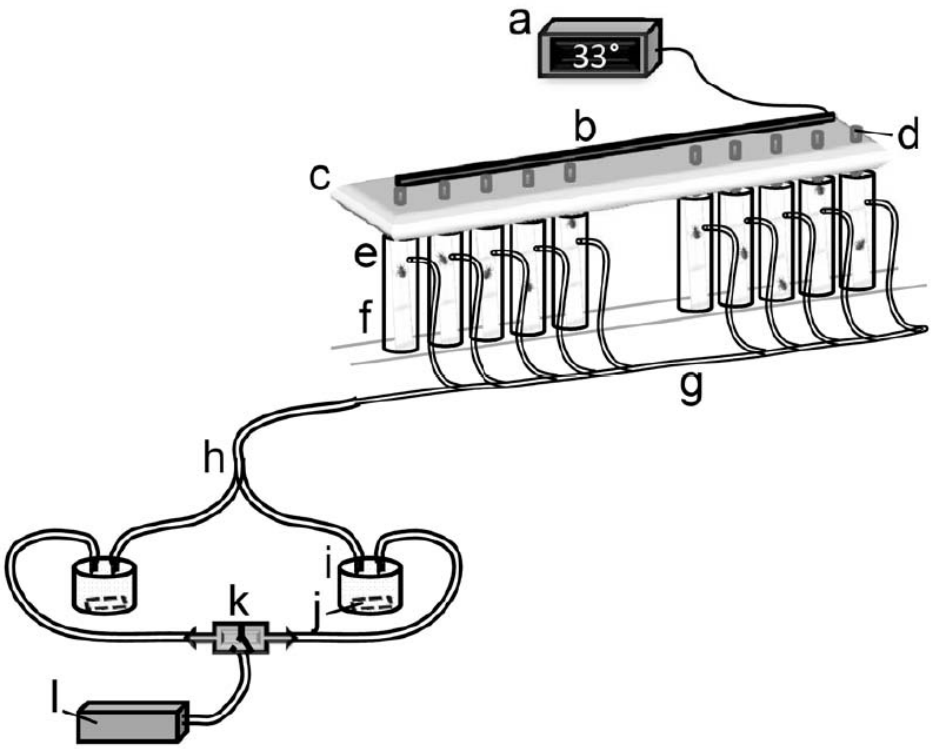

Figure 1.1: Artificial feeder used in the appetitive conditioning procedure. It allows the pairing of the presentation of an odorant [L-lactic acid, conditioned stimulus (CS)] with a heat [unconditioned stimulus (US)] induced blood reward (associative conditioning). a, Thermostat; b, electric resistance; c, isolated aluminium plate; d, blood container; e, insect container; f, filter paper; g, air supply system; h, silicone tubing; i, glass bottle; j, filter paper; k, solenoid valve; l, air pump.

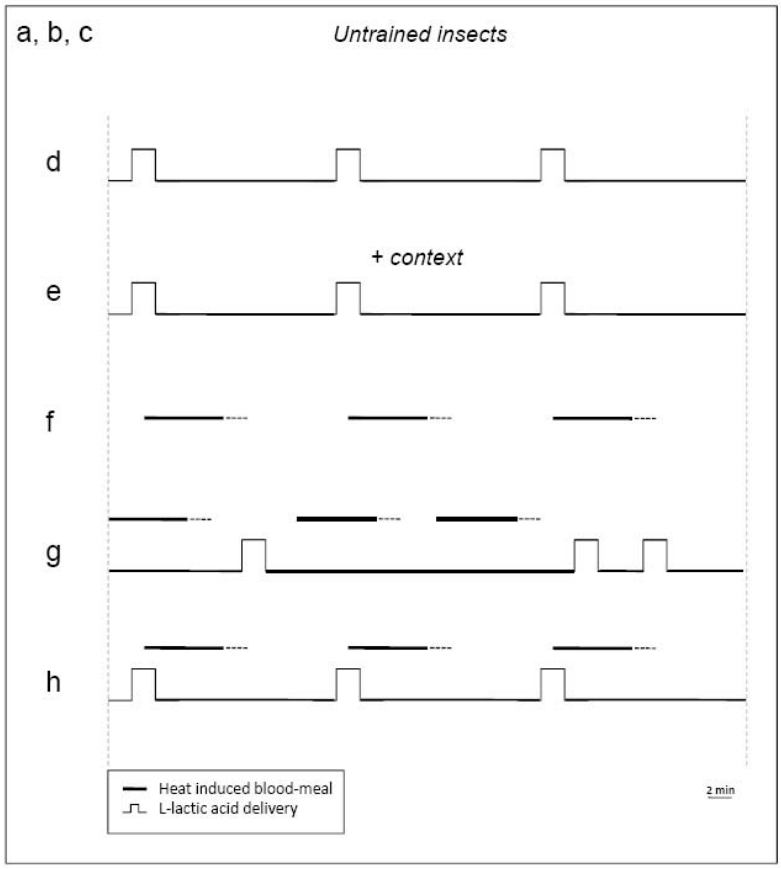

Figure 1.2: Sequence of event delivery (i.e. US, CS and inter-trial interval) during training sessions of the different experimental groups: (a) neutral control (clean air); (b) positive control (yeast); (c) L-lactic acid control; (d) CS only; (e) CS context; (f) US only; (g) unpaired US-CS; and (h) appetitive conditioning (paired US-CS). All training sessions had the same duration, i.e. 45 min. Dotted lines refer to the variability in feeding duration within a group.

to feed until emptying the feeding blood tube and were then removed from the artificial feeder. Only bugs that took the whole amount of blood were kept and maintained in the same experimental room until the next trial; unfed or partially fed bugs were discarded. Trials were separated by 15 min. This inter-trial interval (ITI) was necessary for the bugs to reach the blood container, complete the 15 µl partial blood meal (i.e. approximately 2 to 3 min) and be motivated to feed again. In other words, ITI length was set in order to maximise the feeding success of each trial.

Conditioned bugs were submitted to three trials and thus allowed to feed on a total of 45 µl of blood during the whole training session. This amount represents approximately 26 % of a full meal (mean = 166 mg) and was considered to adequately reward the bugs, but kept their motivation to feed throughout the experiment high. The experimental procedure is summarised in Fig. 2.2.

Testing procedure: the olfactometer. To compare the response to LA of control, untrained and trained bugs, an olfactometer was used. It consisted of an enclosed Y-maze made of Plexiglas® (Fig. 1.3). The angles between the arms were 120°C. Two of the arms were connected to air inlets. The air streams were generated by two independent air pumps with independently controlled flow rates (flow rate = 1.18 $cm^3.s^{-1}$, air speed = 4.2 $cm.s^{-1}$). Each air current flowed through a glass bottle (250 ml) containing a piece of filter paper (2.5 cm^2) on which either distilled water or LA diluted in distilled water (100 µg LA 50 $µl^{-1}$ water) was applied. In order to avoid any pressure increase inside the maze, the third arm (starting arm) was connected to an air exhausting pump with a controlled flow rate of 3.8 $cm^3.s^{-1}$. All connections were made using silicone tubing (0.6 cm diameter).

The scene was illuminated by a red lamp (12 $µW.cm^{-2}$), as bugs are less sensitive to red light than to other wavelengths (Reisenman and Lazzari, 2006). Temperature (25 °C) and relative humidity (40-60 %) remained constant throughout all experiments. In order to avoid environmental biases, the position of the stimulus and control currents were randomly exchanged.

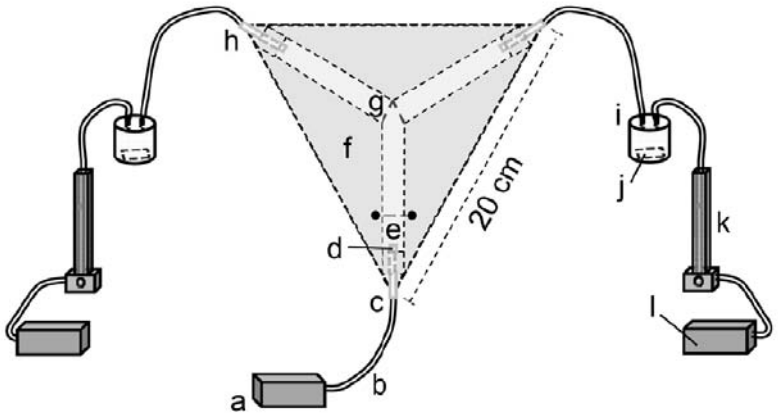

Figure 1.3: Olfactometer designed for the analysis of the olfactory orientation of triatomines. a, Air-extracting pump; b, silicone tubing; c, glass tube; d, familiarisation room; e, nylon door; f, Plexiglas® olfactometer; g, decision line; h, air inlet; i, glass bottle; j, filter paper; k, flow meter; l, air pump.

At the beginning of an experiment, one bug was placed in a starting chamber located at the extremity of the starting arm, and closed by a nylon door (Fig. 1.3). After 1 min of familiarisation, the door was opened. Led by its positive anemotaxis (Barrozo *et al.*, 2003), the insect walked along the starting arm and, at the bifurcation, could choose to follow one of the two air streams, one bearing the stimulus and the other only clean air, by entering into one of the two choice arms. We considered the first choice made by bugs, when they crossed an arbitrary decision line at the entry of each arm. Bugs that did not choose or did not leave the starting chamber were considered as not responding.

The following groups of insects were tested:

(1) Neutral control. Naive bugs were tested in the olfactometer while delivering two clean air currents, in order to test for experimental biases (n= 39).

(2) Positive control. Naive bugs were exposed to a clean air current versus baker's yeast fermentation odour (*Saccharomyces cerevisiae*) (n= 22). Yeast was introduced in a glass bottle containing water (5 ml) and sugar (2.5 g), inducing the release of CO_2 and other components (Williams *et al.*, 1981) that are attractive to triatomines

(Guerenstein *et al.*, 1995; Lorenzo *et al.*, 1998). The aim of this test was to determine whether the apparatus was able to reveal an oriented response of bugs.

(3) L-Lactic acid control. Naive bugs were confronted to a clean air current versus a LA-loaded stream (n= 22), to which, according to previous work (Barrozo and Lazzari, 2004b), they are supposed to be indifferent.

(4) CS only. Non-rewarded bugs (n= 17) were pre-exposed to LA in a distinct plastic container, following the same procedure as the conditioned bugs but without being pre-exposed to the artificial feeder, heat or food. Bugs were tested in the olfactometer 15 min later for their response to a clean air current versus a LA-loaded stream.

(5) CS context. Non-rewarded bugs (n= 18) were put inside the training containers in the artificial feeder and pre-exposed to LA only, following the same procedure as conditioned bugs except for the absence of heat and food. Bugs were tested in the olfactometer 15 min later for their response to a clean air current versus a LA-loaded stream.

(6) US only. Bugs (n= 12) were submitted to the same procedure as conditioned bugs without being exposed to LA during the training phase. Bugs were tested in the olfactometer 15 min after their last partial blood meal for their response to a clean air current versus a LA-loaded stream.

(7) Unpaired US-CS. During the training session, bugs (n= 17) were stimulated by heat (US) and allowed to feed on partial blood meals. They also were exposed to LA (CS), without contingency with the US. For each trial, the sequence of events (i.e. US, CS and two half- ITI) was randomly generated for each individual. An example of training sequence is given in Fig. 1.2. As a consequence, the duration of the training session was identical to the appetitive conditioning group (see below) and insects were submitted to the same amount of stimulation. The only differences with the appetitive conditioning group are the absence of contingency and the random presentation of the US and CS. Bugs were tested in the olfactometer 15 min after the training session for their response to a clean air current versus a LA-loaded stream.

(8) Appetitive conditioning (paired CS-US). Bugs (n= 18) were trained to the contingency between LA (CS), heat (US) and blood reward. Bugs were tested in the olfactometer 15 min later for their response to a clean air current versus a LA-loaded stream.

Binary data collected in the olfactometer were analysed and all statistical tests were computed using R software (R Development Core Team, 2010). Comparisons were performed by means of the exact binomial test (α= 0.05). For each group, the choice of the bugs in the olfactometer was compared to a random distribution of 50 % on each arm of the maze. We also compared the percentage of bugs that remained in the starting chamber of the maze by means of a chi-square test.

1.3 Results

From their initial position in the starting chamber of the Y-maze olfactometer, bugs displayed different behavioural responses according to their respective training experience. Results are depicted in Fig. 1.4. Naive untrained bugs of the neutral control group (clean air *versus* clean air) revealed no bias in the olfactometer or in the experimental room. Indeed, 51.28 % of the bugs chose one arm and 48.72 % chose the other. These results were not significantly different from a random distribution of 50 % in each arm (binomial exact test, p= 0.97). When they were confronted with a clean air current versus a current loaded with yeast odour, the naive bugs of the positive control group chose preferentially the arm delivering the odour of baker's yeast fermentation (81.82 %). Thus, the device proved to be adequate for revealing an oriented response of *R. prolixus*. The difference between the distribution of bugs in this case and a random distribution was significantly different (binomial exact test, p= 0.002). A third group of naive bugs was confronted to an air current loaded with LA *versus* a clean air current. Bugs of this group did not show any oriented response regarding LA, i.e. attraction or repulsion (LA, 54.55 %; clean air, 45.45 %) and their distribution in the maze was not significantly different from a random distribution (binomial exact test, P= 0.42). This is in line with the results of Barrozo and

Lazzari (Barrozo and Lazzari, 2004b).

Two groups of bugs were pre-exposed to LA. The CS-only group, pre-exposed to LA but not to the experimental context (i.e. setup, containers, etc.), did not display any oriented distribution (LA, 44 %; clean air, 56 %; binomial exact test, $p = 0.61$). Bugs of the CS-context group exhibited a small but non-significant preference for clean air current (clean air, 66.67 %; towards LA, 33.33 %; binomial exact test, $p = 0.23$, n.s.). In other words, pre-exposition to LA did not induce any significant change in the behavioural response to LA during a subsequent exposition. This was true when bugs were pre-exposed to LA either with or without being submitted to the same manipulations as the trained group.

Bugs of the US-only group were pre-exposed to heat and fed on three blood meals (15 µl each), an were manipulated in the same way as the conditioned bugs but without being exposed to LA. When tested in the olfactometer, they displayed a non-oriented (i.e. random) distribution (50 % in each direction, binomial exact test, $p = 0.98$), revealing no effect of the ingestion of blood on the behavioural response to LA.

For the unpaired US-CS group, the US and the CS were delivered in a random order during the training session. Bugs that were submitted to this training procedure displayed a random orientation during the test (binomial exact test, $p = 0.63$). The arm equipped with the LA-loaded air stream was picked out by 41.2 % of the bugs, whereas 58.8 % of them chose to walk toward the clean air current. These results discard any cumulative effect of US and CS presentation in absence of contingency.

The last group of bugs was exposed to the contingency of a LA-loaded air current and heat-induced, controlled blood meal (Fig. 1.2). The majority of bugs belonging to this appetitive conditioning group chose the arm delivering the LA-loaded current (72.73 %; binomial exact test, $p = 0.04$), revealing a clearly oriented behavioural response towards LA following the training procedure, i.e. an attraction for the LA-loaded air stream.

It should be mentioned that in all tests, most bugs left the starting chamber of the olfactometer and entered into one of the decision arms. A small proportion of bugs did

not respond and remained in the starting chamber (approximately 18.4 %). This proportion was similar across the groups (chi-square test, $p = 0.81$), and was excluded from the statistical analysis.

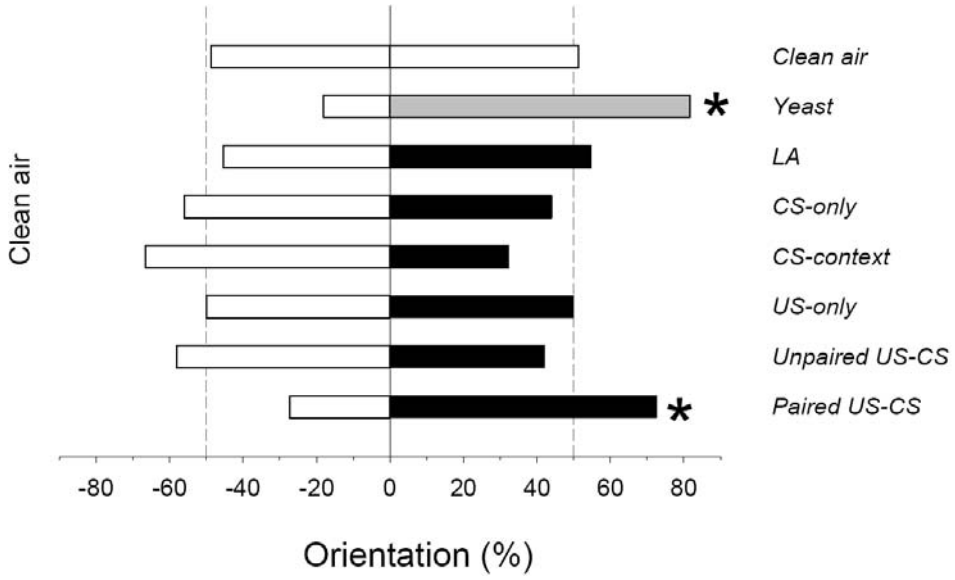

Figure 1.4: Orientation response of *Rhodnius prolixus* larvae tested in the olfactometer and confronted with two air currents: clean air (white bars) and air loaded with either volatiles produced by baker's yeast (grey bar) or L-lactic acid (black bars). Orientation is represented by the percentage of bugs choosing each of the two test arms. Each bar represents an experimental group: clean air, neutral control group ($n = 39$); yeast, positive control group ($n = 22$); LA, L-lactic acid control group ($n = 22$); CS only, CSonly group ($n = 17$); CS context, CS-context group ($n = 18$); US only, US-only group ($n = 12$); unpaired US-CS, unpaired US-CS group ($n = 17$); paired US-CS, appetitive conditioning group ($n = 18$). Asterisks indicate distributions that are significantly different from random ($P < 0.05$).

1.4 Discussion

Until now, no evidence had been obtained regarding the ability of blood-sucking bugs to learn and, more generally, only few experimental data are available on the cognitive abilities of haematophagous insects, not all of them being conclusive (Alonso *et al.*, 2003; Alonso and Schuck-Paim, 2006). Concerning *R. prolixus*, only negative results have been reported (Abramson *et al.*, 2005; Aldana *et al.*, 2008). In the present study, the accumulated knowledge on *R. prolixus* biology was exploited in order to adapt a conditioning

procedure to its haematophagous way of life and to assess whether their behavioural response to a neutral odour could be modified by individual experience. The results of our experiments reveal that *R. prolixus* is able to associate a neutral stimulus with a positive (food) reinforcement, turning the odour attractive. In a companion paper we show that the same neutral odour can also be associated with a negative reinforcement (i.e. punishment represented by mechanical perturbation), making it repellent (Vinauger *et al.*, 2011b).

The association of LA (CS) with heat (US) as a predictor of food resulted in a modification of the bugs' originally neutral response to LA to a positive response. This behavioural change can be interpreted as appetitive conditioning, and indicates that *R. prolixus* are able to adapt their innate response to natural (host-associated) stimuli.

In our experiments, the groups pre-exposed to LA (i.e. when the odour was not associated to food) showed a light tendency to avoid the LA-loaded air stream. Because it would be counter-adaptive for bugs pre-exposed to a host odour without further consequences to become repulsed by this compound, we can assume that this aversion was due to handling. As a consequence, the aversive effect of handling may also be present during the appetitive conditioning procedure because bugs were manipulated in an identical way. Despite this bias, significant attraction to LA was revealed in conditioned bugs' choice (Fig. 1.4), and the consistent random choice across the different control groups allows us to discard any possible effect of one of the two stimuli (i.e. CS or US) acting alone, the cumulative effect of US and CS, as well as a potential effect of pre-exposure to LA.

It should be noted that most work on insect olfactory conditioning has been performed on employing sugar-feeding species, such as honeybees, flies and butterflies. In such cases, a stimulation of taste receptors on the antennae, legs or other body parts is sufficient to trigger proboscis extension and feeding on a drop of sugar solution (e.g. Bitterman *et al.*, 1983). This is not case for blood-sucking insects, which need to pierce the host skin before gaining access to the blood, e.g. from inside blood vessels or from a wound (Lazzari, 2009). In the case of *R. prolixus*, proboscis extension is not induced by blood components, but in response to heat stimulation (Lazzari and Núñez, 1989; Flores and Lazzari, 1996). So, the

US (heat) can be detached from the reward (blood), which is not case in nectar-feeding insects, for which sugar can represent both the US and the reward.

Thus, rewarding haematophagous insects requires providing blood at a temperature corresponding to that of a potential host. Another important point to be taken into account is that the consumption of blood is known to induce a change in the physiological and motivational status of the insect (Bodin *et al.*, 2009a). We avoided this in our experiments by providing small controlled amounts of blood as positive reinforcement. Thus, our experimental design allowed us to apply Pavlovian conditioning procedures to triatomine bugs, overcoming the major difficulties associated with haematophagy, i.e. providing insects with access to the reward and maintaining a high motivational status during the conditioning procedure. A further pre-requisite was to ensure a contingency between the US and the CS while limiting handling and perturbations of insects. The device that we set up allowed us to deliver a continuous air current, to precisely control the duration of LA stimulation and to provide a partial blood meal within the same apparatus. Despite these precautions, it turned out that handling of the bugs' containers resulted in a trend (also present in the second test in the companion paper) that may indicate an effect on bugs.

As indicated above, this was not the first attempt to assess whether Pavlovian conditioning could modify the behaviour of *R. prolixus*. Nevertheless, previous work did not succeed in evincing any kind of olfactory conditioning in these bugs. Comparing our experimental approach to previous ones may shed some light on the reasons behind these contradictory results, and provide some insights for future work. In their foray, Abramson *et al.* (2005) and Aldana *et al.* (2008) also paired an olfactory CS with a thermal US using an artificial feeder. The major differences with our work lie in the way they developed their experimental design. First, insects were not allowed to feed on blood, but on saline solution. As no indication about the addition of phagostimulants is provided, saline solution may not constitute a true reward for bugs, given that these compounds are known to be indispensable for food recognition by *R. prolixus* (Friend and Smith, 1977). Secondly, the odours used as potential CS were almizcle, cinnamon, citral, ruda, raccoon and buck-lure

[the rationale of the choice being that "*Almizcle and ruda are common colognes used by Venezuelan men; cinnamon has been used in studies of learning by honey bees* (...). *The animal odours were selected because raccoon and buck are not found in Venezuela*" (Abramson *et al.*, 2005)]. It is worth mentioning that these are complex blends of which perception by the bugs' sensory system has never been tested. As a consequence, one cannot be sure that the CS or the reward was perceived as such by the bugs. In our experiments, we have chosen to employ a volatile whose purity and concentration are known, as well as the ability of the bugs to perceive it, but to which they do not exhibit any oriented response when presented alone (Barrozo and Lazzari, 2004a; Barrozo and Lazzari, 2004b) (Fig. 1.4). This feature allowed us to use it in both appetitive and aversive contexts, as can be seen in Vinauger *et al.* (Vinauger *et al.*, 2011b).

The ability of *R. prolixus* to learn can be seen as a response to the strong selection pressures acting on haematophagous insects. Indeed, learning and memory could improve the capacity of blood-sucking insects, by narrowing their search, aiding in the selection of targets, conserving energy as well as enhancing their resource-locating efficiency (McCall and Kelly, 2002). It should be remembered that for these bugs, a host plays the double role of prey and predator (Lazzari, 2009). Although it is adaptive to recognize and remember any cues from high-quality resources, it seems equally necessary to be able to avoid the most defensive hosts (McCall and Kelly, 2002). It would be as adaptive for bugs to be able to associate particular host signals with active defensive behaviour. This will be the step developed in part II (Vinauger *et al.*, 2011).

1.5 Acknowledgements

The authors are indebted to F. Guerrieri and the staff members of the Neuroethology group for fruitful discussions, to C. Lahondère and F. Dupuy for critical reading of drafts and to C. Labrousse for technical support with insect rearing. This work received support from the CNRS, the University of Tours and the Agence Nationale de la Recherche (ANR-08-MIE-007, EcoEpi), France. This study was performed as part of the PhD work of C.V. at the University of Tours, under the supervision of C.R.L. and with the support of the

French Ministère de la Recherche.

II. Apprentissage olfactif aversif

Après avoir démontré la possibilité de modifier la réponse comportementale de *R. prolixus* à une odeur par conditionnement appétitif, nous avons cherché à savoir si la même odeur pouvait être associée avec la perspective de recevoir une punition. Comme évoqué précédemment, pour ces insectes, l'hôte peut jouer le rôle de proie, c'est-à-dire de source de nourriture, aussi bien que le rôle de prédateur. Être capable d'associer l'odeur d'un hôte avec son comportement défensif permettrait donc aux triatomines d'éviter les hôtes les plus défensifs, à une distance où ces derniers ne représentent pas encore un réel danger. Cette capacité permettrait aux insectes de maximiser les chances d'obtenir un repas sanguin tout en minimisant les risques d'être blessé ou tué au cours de l'opération.

Nous avons donc adapté la procédure expérimentale développée dans l'étude précédente de façon à pouvoir apparier la stimulation olfactive (CS) avec la présentation d'un choc mécanique contrôlé (US), permettant de mimer le comportement défensif d'un hôte. L'odeur utilisée est la même que lors du conditionnement appétitif, l'acide L-lactique. En revanche, s'agissant de conditionnement aversif, l'olfactomètre en Y utilisé dans l'expérience précédente n'apparaissait plus comme adapté. En effet, depuis leur position de départ, les insectes percevraient une odeur potentiellement aversive, sans avoir la possibilité de fuir. Nous nous attendrions donc à observer une augmentation de la proportion d'insectes ne quittant pas la position de départ. Pour palier cela, la réponse comportementale des insectes a été testée dans un compensateur de locomotion permettant aux individus de marcher vers le stimulus ou dans la direction opposée.

Tandis que l'acide lactique n'induit pas de réponse orientée chez les individus naïfs,

les individus entrainés évitent l'odeur, choisissant de s'orienter préférentiellement dans la direction opposée à la source d'acide lactique. Les groupes contrôles ont permis d'écarter les effets potentiels d'un stimulus (CS ou US) agissant seul ou l'action cumulée des stimuli (CS et US) en l'absence de contingence (CS-US non-appariés).

Ces résultats constituent la première démonstration qu'une même odeur de l'hôte peut être utilisée par les insectes vecteurs de maladies pour apprendre à reconnaitre soit un hôte sur lequel s'alimenter soit un hôte potentiellement défensif. De plus, dans ces deux études (conditionnement appétitif et aversif) les individus testés ont été capables d'utiliser une information acquise dans un contexte expérimental donné (dispositifs d'entrainement), dans un contexte différent (dispositifs de test).

L'ensemble des résultats de ces deux articles sont discutés en intégrant l'impact des processus mis en évidence dans le choix des hôtes.

Article publié: Vinauger, C., Buratti, L. & Lazzari, C. R. (2011b). *Journal of Experimental Biology* **214**: 3039-3045.

Chapitre 2

Learning the way to blood: first evidence of dual olfactory conditioning in a bloodsucking insect, *Rhodnius prolixus*. Part II: Aversive learning

Abstract. After having demonstrated that blood-sucking bugs are able to associate a behaviourally neutral odour (L-lactic acid) with positive reinforcement (i.e. appetitive conditioning) in the first part of this study, we tested whether these insects were also able to associate the same odour with a negative reinforcement (i.e. aversive conditioning). Learned aversion to host odours has been repeatedly suggested as a determinant for the distribution of disease vectors among host populations. Nevertheless, no experimental evidence has been obtained so far. Adapting a classical conditioning approach to our haematophagous model, we trained larvae of *Rhodnius prolixus* to associate L-lactic acid, an odour perceived by bugs but behaviourally neutral when presented alone, with a mechanical perturbation (i.e. negative reinforcement). Naive bugs and bugs exposed to CS, punishment, or CS and punishment without contingency remained indifferent to the presence of an air stream loaded with L-lactic acid (random orientation on a locomotion compensator), whereas the groups previously exposed to the contingency CS-punishment were significantly repelled by L-lactic acid. In a companion paper, the opposite, i.e. attraction, was induced in bugs exposed to the contingency of the same odour with a positive reinforcement. These constitute the first pieces of evidence of olfactory conditioning in triatomine bugs and the first demonstration that the same host odour can be used by insects that are disease vectors to learn to recognize either a host to feed on or a potentially defensive one. The orientation mechanism during repulsion is also discussed in light of our results.

Key words: associative learning, Chagas disease, host selection, haematophagous, Triatominae.

2.1 Introduction

The ability of insects as disease vectors to learn and remember could have important consequences in the epidemiology of the diseases that they transmit. These abilities could improve the capacity of blood-sucking insects to select hosts more efficiently (McCall and Kelly, 2002). Targeting the most vulnerable hosts and avoiding the most defensive ones results in an heterogeneous distribution of biting (e.g. some people being more bitten than others) in host populations, affecting the circulation of parasites among the population members (Kelly *et al.*, 1996; Kelly, 2001).

As previously highlighted by Alonso and Schuck-Paim (2006), developing a deeper comprehension of the learning abilities of disease vectors could help us to better understand how individual experience influences vectors' hosts choices and how it might affect their potential for disease transmission. In recent literature, examples of learning-improved host selection can be found, particularly regarding mosquitoes. Tomberlin *et al.* (2006) brought experimental evidence of associative olfactory learning in *Culex quinquefasciatus* adults obtained under controlled laboratory conditions. In less controlled but more ecologically relevant ways, Charlwood *et al.* (1988) have shown that *Anopheles farauti* remembered home range, and Mwandawiro *et al.* (2000) reported that *Culex vishnui* displayed higher feeding rates when they were exposed to host species on which past blood-feeding was successful. The ability of *Rhodnius prolixus* to learn to associate a neutral host odour with a heat-associated blood meal has been demonstrated in part I of the present study (Vinauger *et al.*, 2011a).

It is worth noting, however, that for blood-sucking insects a host plays the double role of prey and predator (Lazzari, 2009). They take food from their host but, in doing so, expose themselves to the possibility of being hurt or killed by host grooming or defensive

behaviours. Thus, although it is adaptive to learn and remember any cues paired with high-quality hosts, it also seems necessary to be able to learn to avoid the most defensive ones (McCall and Kelly, 2002). This could be accomplished by associating particular host signals with a high level of defensive behaviour. Because olfaction allows the insect to locate and recognize their host from a distance where the hosts are not yet a threat (Lazzari, 2009), odours represent, as developed in Vinauger *et al.* (2011a), a good target for learning. In previously published work, we have shown that the same host odour may either attract or repel haematophagous insects according to their physiological state (Bodin *et al.*, 2009a), but nothing is known about the influence of experience on the significance that a given odour may have on a particular individual. Thus, the aims of the present study were to: (1) investigate whether a haematophagous insect is able to associate a host odour with its defensive behaviours and (2) assess whether the same host odour can be used in both appetitive and aversive conditioning, i.e. to test whether the same neutral odour is able to become attractive or repellent for haematophagous insects, depending on their individual experience.

2.2 Material and Methods

As in the first part of this study (Vinauger *et al.*, 2011a), we used triatomine bugs as experimental models and tested whether *Rhodnius prolixus* Stål 1859 larvae are able to associate the same behaviourally neutral (but perceived) odour that was used in Vinauger *et al.* (2011a) with the prospect of being hurt or killed by the host defensive behaviour. Here, the choice of L-lactic acid (LA) as the conditioned stimulus (CS) comes fully into play because this compound is both a biologically relevant host odour and a behaviourally neutral one that allows us to investigate the consequence of its pairing with both positive and negative reinforcement. Furthermore, because this study deals with aversive conditioning, the use of a Y-maze olfactometer (as used in Vinauger *et al.*, 2011a) would be incongruous. Indeed, from their initial position in the starting chamber, insects would perceive

the potentially aversive odour without having the possibility to walk away from it. This means that we would expect an increase in the number of insects remaining in the starting chamber, leading to the impossibility of clearly quantifying any oriented behaviour. Thus, we used an experimental paradigm allowing us to pair LA (CS) with a mechanical perturbation (unconditioned stimulus; US) before testing the behavioural responses of insects in an open-loop locomotion compensator.

2.2.1 Insects

As our experimental subject we chose *R. prolixus*, a classical model in insect physiology and also a main vector of Chagas disease in Central and South America (Dias *et al.*, 2002). Bugs were reared in the laboratory under a 12:12h light:dark illumination regime, at 28°C and 60-70 % relative humidity. Insects were fed weekly with sheep heparinised blood, using an artificial feeder (Nunez and Lazzari, 1990). Fifth-instar larvae that had just moulted were isolated in individual plastic containers and starved until being tested, 15 days after their moult. The experiments were carried out during the first hours of the scotophase (i.e. the dark period, or night-time, of the light cycle), because triatomines display a peak of activity throughout this period, which corresponds to the moment insects leave their refuges to search for host-emitted cues (Lazzari, 1992).

2.2.2 Conditioning procedure

For these experiments we used LA as the CS. This volatile is emitted by vertebrates and has been detected on human skin (Acree *et al.*, 1968; Bernier *et al.*, 2000), and was demonstrated to be the major component of perspiration (Braks *et al.*, 1999), excreted at concentrations between 0.5 and 5 $mg.ml^{-1}$ (Eiras and Jepson, 1991; Cork and Park, 1996; Geier *et al.*, 1996). It is perceived by bugs but does not elicit any oriented response (i.e. attraction or repulsion) when presented alone. However, when combined with CO_2, LA lowers the response threshold for this gas and increases the response to fatty acids (Barrozo and Lazzari, 2004a; Barrozo and Lazzari, 2004b). Furthermore, as demonstrated

previously (Vinauger *et al.*, 2011a), *R. prolixus* larvae are able to associate this compound with the possibility of obtaining a blood meal.

Training procedure: pairing CS and mechanical perturbation. In order to investigate whether *R. prolixus* is able to associate this odour with a negative reinforcement, we set up a device allowing us to pair the presentation of LA (CS) with a mechanical shock (US). We chose mechanical shock as the punishment to mimic the defensive behaviour of a host and because its contingence with odours has been shown to induce olfactory conditioning in other insects (Mery *et al.*, 2007).

Rhodnius prolixus were placed in a plastic jar supplied with an air delivery system similar to that previously described (Vinauger *et al.*, 2011a), delivering LA-loaded air or clean air. The mechanical shock was delivered to groups of five to seven bugs by a low-speed electric motor (approximately 240 rotations.min^{-1}; i.e. 4 Hz), on the axis of which 15 paper strips (2-3 mm wide; 4 cm length) were fixed (Fig. 2.1). This way, we were able to submit the insects to a mechanical perturbation without hurting them. Indeed, no effects on subsequent moults or any increased mortality was observed.

Before the training session began, insects were allowed to familiarise themselves with the plastic jar for 2 min, without stimulation. After this time, an air current loaded with LA was delivered for 1 min and then the LA current was paired with the mechanical shock for an additional 1 min. Bugs were submitted to five trials, separated by 5 min inter-trial intervals (ITIs). The experimental procedure is summarised in Fig. 2.2.

Testing procedure: use of a locomotion compensator. To test for aversive conditioning, we used a locomotion compensator to obtain the walking paths of control and trained bugs when stimulated by an air current loaded with LA versus a clean air current (Fig. 2.3). The trajectories of the bugs were recorded in an open-loop design for translation, and their spatio-temporal components were analysed, as described below (see Data analysis).

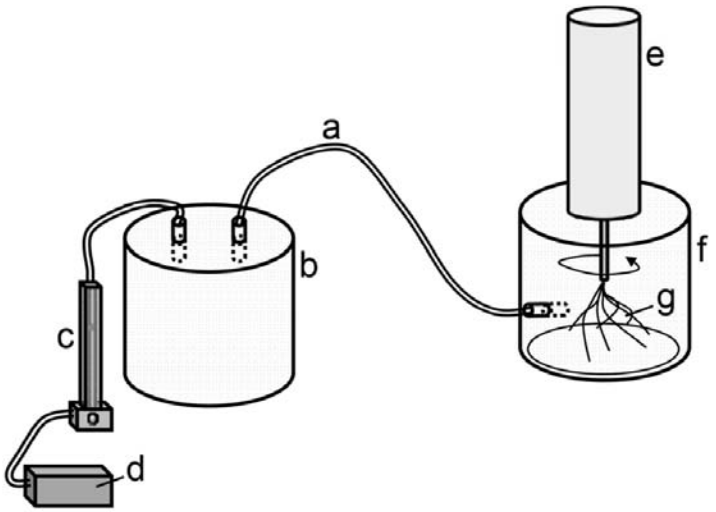

Figure 2.1: Experimental device allowing either unpaired or paired presentation of L-lactic acid [LA; conditioned stimulus (CS)] and a mechanical perturbation [unconditioned stimulus (US)]. a, Silicone tubing; b, glass bottle containing either distilled water or LA (100 µg 50 μl^{-1} distilled water) on a piece of filter paper (2.5 cm^2); c, flow meter; d, air pump; e, electric motor; f, insect container; g, paper strips (0.2-0.3 cm wide, 4 cm long).

Before the beginning of each test, each bug remained in still air on the locomotion compensator for 2 min so it could familiarise itself with the experimental situation, after which two air streams coming from opposite directions were presented for 3 min. The assays were monitored from the outside of the experimental room by means of an infrared-sensitive camera (Conrad, Lille, France) equipped with an array of infrared LEDs (emission 900 nm). This light illuminated the scene without being perceived by the bugs (Reisenman *et al.*, 1998; Reisenman and Lazzari, 2006). Because triatomines exhibit spontaneous anemotaxis to odourless air streams under these conditions (Barrozo *et al.*, 2003), a simultaneous discrimination bioassay was conducted, similar to that used previously (Barrozo *et al.*, 2004; Barrozo and Lazzari, 2004a; Barrozo and Lazzari, 2004b; Bodin *et al.*, 2008; Bodin *et al.*, 2009a; Bodin *et al.*, 2009b).

Individual bugs were exposed to two different horizontal air streams (180 °): one was loaded with LA whereas the other delivered clean air only (test versus control). Thus, each bug could choose either to walk towards one of the two streams or to exhibit a non-

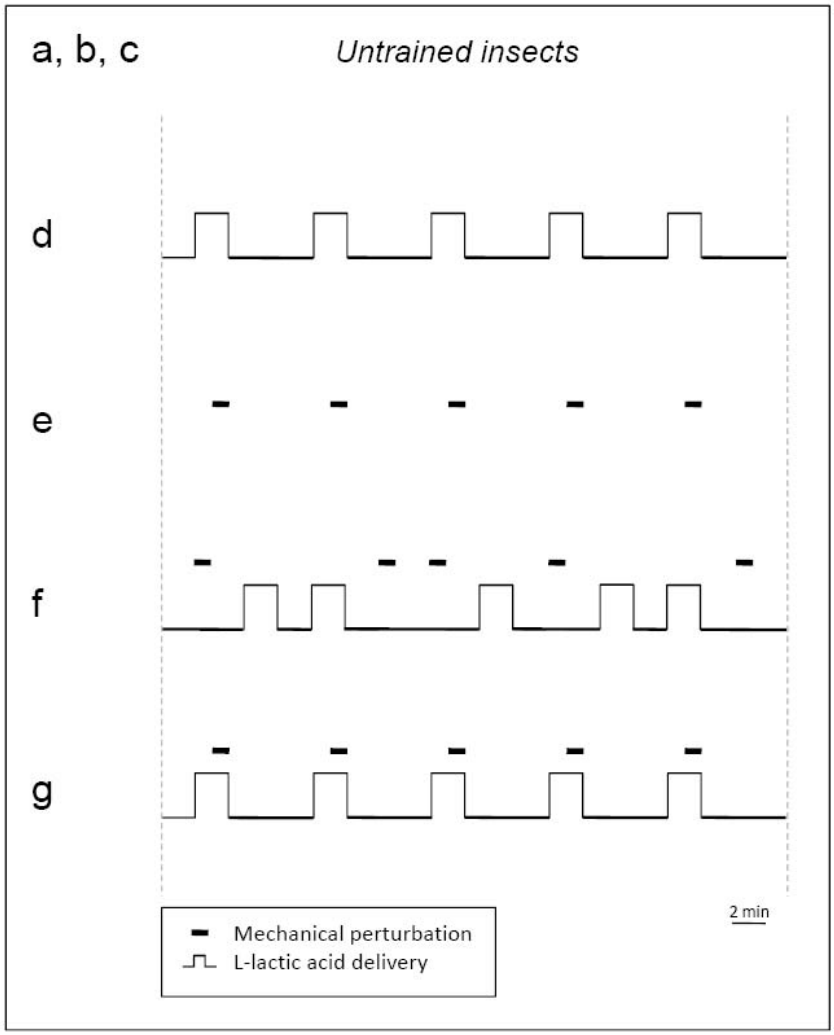

Figure 2.2: Sequence of events (i.e. US, CS and inter-trial interval) during training sessions of the different experimental groups. (a) Neutral control group; (b) positive control group; (c) L-lactic acid control group; (d) CS-only group; (e) US-only group; (f) unpaired US-CS group; (g) aversive conditioning group. All training sessions had the same duration, i.e. 35 min.

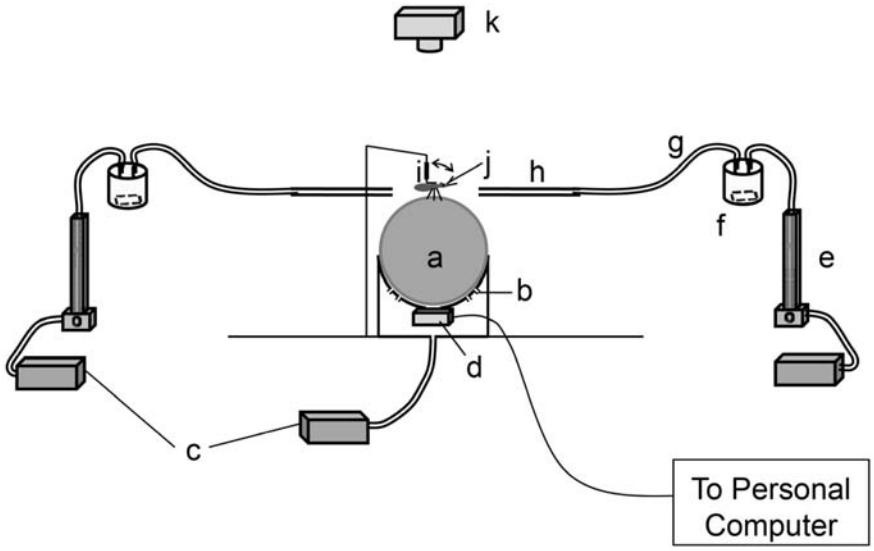

Figure 2.3: Locomotion compensator designed for the analysis of the olfactory orientation of triatomines. a, Hollow Styrofoam sphere; b, sphere support with air inlets; c, air pumps; d, optical sensor; e, flow meter; f, glass bottle containing a piece of paper on which the odour can be deposited; g, silicone tubing; h, glass tube; i, 360 ° rotating bug support; j, experimental bug, tethered by its thorax with adhesive tape; k, infrared-sensitive camera equipped with infrared LEDs (emission 900 nm).

oriented behaviour, i.e. to walk randomly. Both air streams were blown over the bugs through glass tubes (0.6 cm inner diameter, 14 cm length), placed 3 cm from the bugs, at constant velocity (4.2 $cm.s^{-1}$), temperature (25 ± 2°C) and relative humidity (40 ± 5 %). The release of LA was achieved as previously described (see Vinauger *et al.*, 2011a). To avoid eventual environmental biases, the positions of the stimulus and the control air streams were changed randomly throughout the experiments.

The experimental groups were as follows:

(1) Neutral control group. Naive bugs (n= 20) were exposed to two opposite clean air currents in order to test for any experimental bias.

(2) Positive control group. Naive bugs (n= 16) were exposed to a clean air current *versus* baker's yeast fermentation odour (*Saccharomyces cerevisiae*). Yeast was introduced in a glass bottle containing water (5 ml) and sugar (2.5 g), inducing the release of

CO_2 and other components (Williams *et al.*, 1981) that are attractive to triatomines (Guerenstein *et al.*, 1995; Lorenzo *et al.*, 1998).

(3) L-Lactic acid control group. Naive bugs (n= 18) were exposed to a clean air current *versus* a LA-loaded current.

(4) CS-only group. Non-rewarded bugs (n= 19) were placed inside the training container and pre-exposed to LA only, after 2 min of familiarisation. Bugs were tested 5 min later on the locomotion compensator for their response to a clean air current *versus* a LA-loaded current.

(5) US-only group. Bugs (n= 18) were placed inside the training container and the mechanical shock was delivered, after 2 min of familiarisation, in the absence of LA. Bugs were tested 5 min later on the locomotion compensator for their response to a clean air current *versus* a LA-loaded current.

(6) Unpaired US-CS group. During the training session, bugs (n= 19) were submitted to the mechanical shock (US) and exposed to LA (CS), without contingency between US and CS. For each trial, the sequence of events (i.e. US, CS and two half-ITI) was randomly generated for each individual (an example of the sequence is given in Fig. 2.2). As a consequence, bugs of this group were submitted to the same amount of stimulation as the conditioned group and their training session also had the same duration. The only differences with the conditioned group were the absence of contingency and the random delivery order of US and CS. Bugs were tested on the locomotion compensator, 5 min after the training session, for their response to a clean air current *versus* a LA-loaded stream.

(7) Aversive conditioning group. Bugs (n= 18) were trained to the contingency between LA (CS) and mechanical perturbation (US). Bugs were tested 5 min later on the locomotion compensator for their response to a clean air current *versus* a LA-loaded current.

Data analysis. The walking paths of bugs on the locomotion compensator were analysed in the following way. The mean walking angle displayed by each bug along the experimental time was computed. The position of the stimulus-delivered current was conventionally designated as 0°, and the control current as 180°. Bugs orienting in a mean direction within the hemisphere centred on 0° were considered to walk in the direction of the LA-loaded current whereas bugs walking in a mean direction within the hemisphere centred on 180° were considered to walk in the opposite direction, i.e. in the direction of the clean air control current.

Once binarised, data were analysed and all statistical tests were computed using R software (R Development Core Team, 2010). Comparisons were performed by means of the exact binomial test ($\alpha = 0.05$). For each group, the choice of the bugs on the locomotion compensator was compared with a random distribution of 50 % in each hemisphere.

To compare attractive and aversive orientation behaviours, we performed further analysis of walking trajectories using circular statistics. Three different tests were applied: the Rayleigh test, the circular range test and the V-test. The rationale behind using three different tests is that each test provides different information. The Rayleigh test determines whether a circular distribution is uniform, focusing on the angular distance between individual pathways; this test was used to verify whether bugs walked randomly. The circular range test calculates the shortest arc of the circle containing the entire set of data and the P-value is computed using the exact distribution of the circular range under the hypothesis of uniformity, i.e. this test was used to determine whether bugs walked in a certain direction, whatever it was. The V-test analyses whether the bearings are randomly distributed with respect to the predicted direction, i.e. whether bugs oriented towards an expected direction, in this case, in the direction of or opposite to the stimulus.

2.3 Results

Naive and untrained fifth-instar larvae confronted with two clean air currents from opposite directions walked randomly in the locomotion compensator and no oriented behaviour was revealed (binomial exact test, $p = 0.51$; Fig. 2.4). Almost 60 % of bugs oriented in one hemisphere and 40 % in the other one; this orientation was not different from a random distribution. This first result revealed that neither the experimental room nor the experimental device presented any bias. In order to be sure that our experimental setup was adapted to reveal an oriented response, we exposed naive bugs of the positive control group to a clean air current *versus* an air current loaded with baker's yeast volatiles. This time, 75 % of the bugs chose the hemisphere corresponding to the yeast odour current. This orientation was significantly different from a random distribution (binomial exact test, $p = 0.03$; Fig. 2.4).

We also determined that the CS alone was not responsible for any oriented behaviour by testing the responses of naive untrained bugs confronted with a clean air current *versus* a LA-loaded current. LA did not evoke any attractive or repulsive response (binomial exact test, $p = 0.24$; Fig. 2.4), confirming one more time the results obtained by Barrozo and Lazzari (Barrozo and Lazzari, 2004b).

In order to discard any effect of one of the two stimuli acting alone, we then pre-exposed two groups of bugs to either LA (CS) or mechanical perturbation (US). Bugs in the CS-only group did not display any oriented distribution (binomial exact test, $p = 0.18$). The response of the group pre-exposed to the US was similar (binomial exact test, $p = 0.12$).

The results of the experiments with the unpaired CS-US group, for which CS, US and ITIs were delivered in a pseudo-random order, indicate that there is not a cumulative effect of CS and US presentation. Bugs of this group walked towards the two hemispheres in similar proportions (53 % walked towards the clean air current and 47 % walked towards the LA-loaded current; binomial exact test, $p = 0.98$; Fig. 2.4).

The last group of bugs was trained by confronting them with the contingency between

LA delivery and mechanical perturbation. After five training trials, when exposed to an LA-loaded current against a clean air current, bugs of the aversive conditioning group did not walk randomly on the locomotion compensator (binomial exact test, p= 0.007; Fig. 2.4), they clearly avoided the LA-loaded current (84 % of them chose the clean air current).

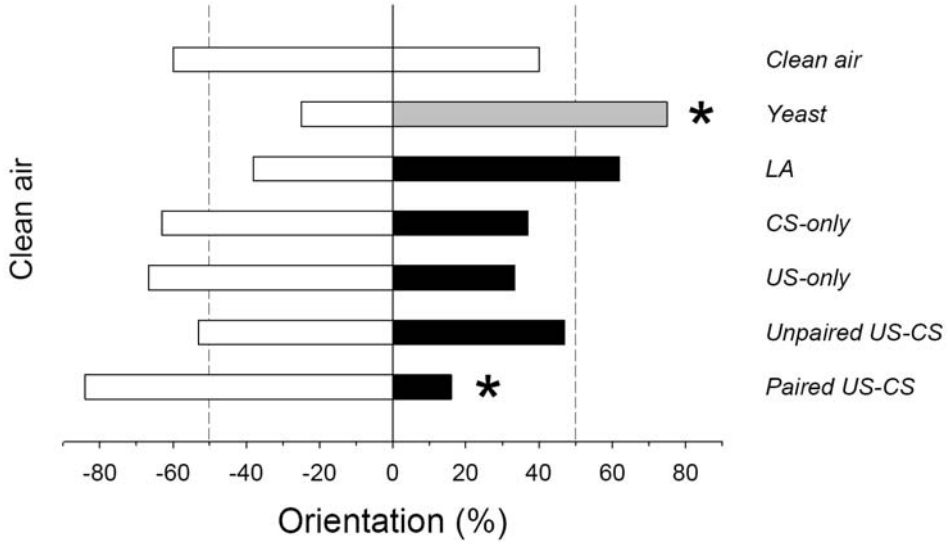

Figure 2.4: Orientation response of *Rhodnius prolixus* larvae tested on the locomotion compensator and confronted with two air currents: clean air (white bars) and either air loaded with volatiles produced by baker's yeast (grey bar) or LA (black bars). Orientation is represented by the percentage of insects choosing each of the two hemispheres. Each bar represents an experimental group: clean air, neutral control group (n= 20); yeast, positive control group (n= 16); LA, L-lactic acid control group (n= 18); CS only, CS-only group (n= 19); US only, US-only group (n= 18); unpaired US-CS, unpaired US-CS group (n= 19); paired US-CS, aversive conditioning group (n= 18). Asterisks indicate distributions that are significantly different from random (P<0.05).

The analysis of walking directions (circular histograms in Fig. 2.5) was performed for the neutral and positive control groups and for the two groups exposed to US and CS (both unpaired and paired). The neutral control group revealed non-oriented walking (Rayleigh, p= 0.41; circular range, p= 0.29; V-test, p= 0.59). The positive control group (i.e. yeast volatiles) revealed a non-uniform distribution of walking angles (Rayleigh, p= 0.04); data are grouped (circular range, p= 0.015) and showed a significant orientation towards the stimulus source (V-test, p= 0.03). The unpaired US-CS group walked in a

random manner (Rayleigh, $p= 0.44$; circular range, $p= 0.98$; V-test, $p= 0.37$). For the aversive conditioning group, the Rayleigh test did not reveal a significant difference between the observed distribution and a uniform one ($p= 0.09$), but the circular range test revealed a significantly grouped distribution of individual orientation angles ($p= 0.0017$). The bugs showed a significant orientation towards 180 ° (i.e. the clean air current and the exact opposite direction of the LA source; V-test, $p= 0.015$). A detailed analysis of the avoiding trajectory revealed, however, that bugs did not walk exactly towards 180 ° . We computed the mean angular disparity between the stimulus direction (0 °) and the absolute value of the walking angle as 113.5 ± 26.2 ° .

It should also be mentioned that during these experiments, only a small proportion of bugs remained immobile on the locomotion compensator (approximately 5 ± 1.8 %). This proportion was similar for all groups (chi-square test, $p= 0.89$) and, as a consequence, those individuals were excluded from statistical analysis.

2.4 Discussion

To deal with the variability of their environment, we expect animals to gather and use information to reduce uncertainty. Learning and memory, defined as an animal's behavioural adjustment based on previous experience and the storage of this experiential information (Lorenz, 1981), are traditionally assumed to be some major contributors to animal fitness. In insects, these abilities are being evinced in a growing number of species.

Until now, no evidence had been obtained demonstrating learning abilities in blood-sucking bugs, and only few conclusive experimental data are available on the cognitive abilities of haematophagous insects in general (Alonso *et al.*, 2003; Alonso and Schuck-Paim, 2006). In the present study, we provide the first experimental evidence that the behavioural response of *R. prolixus* to a neutral host-associated odour can be modified by the bugs' individual experiences to make the odour repellent. Taken together, both the present study and Vinauger *et al.* (2011a) show that *R. prolixus* is able to associate a neu-

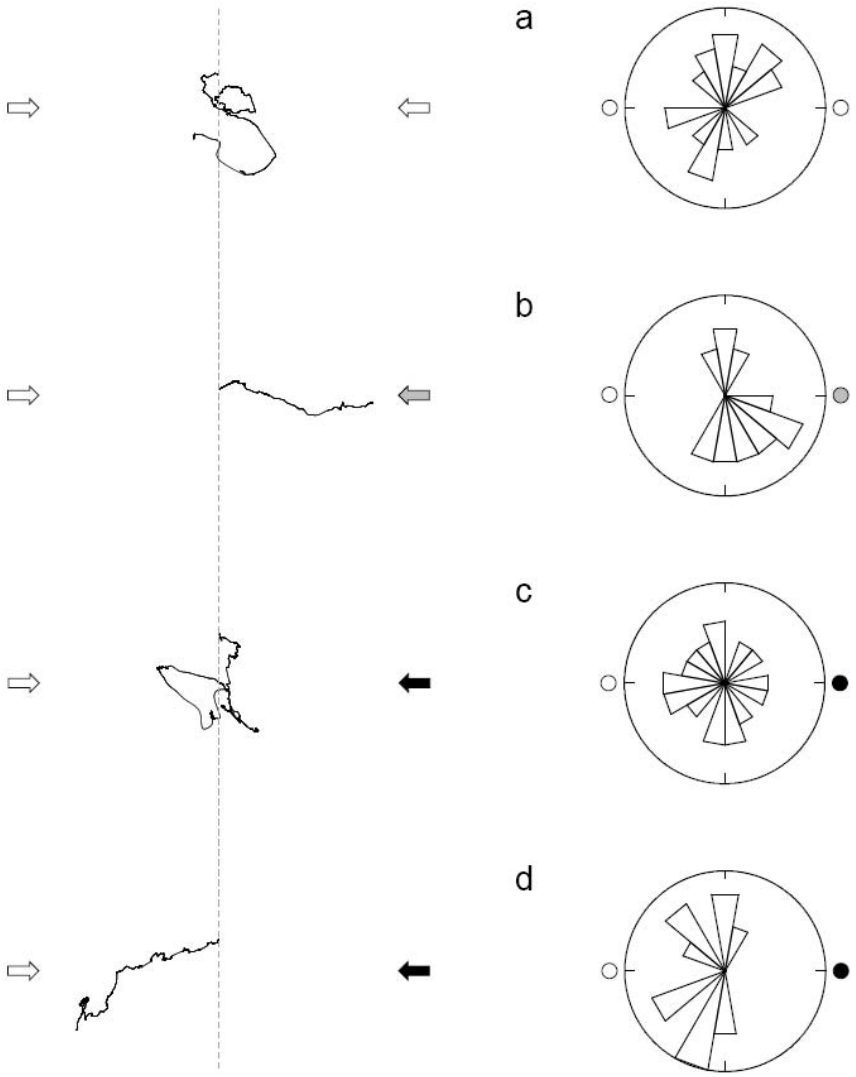

Figure 2.5: (Left) Sample records of pathways described by four *Rhodnius prolixus* larvae when confronted with: (A) still air conditions, (B) control versus yeast-loaded airstream and (C,D) control versus LA-loaded airstream. Dashed line denotes the starting point of the walking bugs. Open arrows indicate the direction of the control air currents and filled arrows denote the direction of either the yeast-enriched (grey) or LA-loaded airstream (black). (Right) Orientation response of *R. prolixus* larvae tested on the locomotion compensator and confronted with two air currents: clean air (open circles) and either air loaded with volatiles produced by baker's yeast (grey circle) or LA (black circles). The circular histograms (rose diagrams) represent the frequency of the angles displayed by the bugs, which is proportional to the area of the wedge (bar width of 20 $^\circ$). Each rose diagram represents an experimental group: (A) neutral control group (n= 20); (B) positive control group (n= 16); (C) unpaired US-CS group (n= 19); (D) aversive conditioning group (n= 18).

tral stimulus either with a positive (food) or a negative reinforcement (mechanical shock), revealing opposite consequences of olfactory experience. The association of LA (CS) with heat (US) as predictor of food and blood (reward) resulted in a modification of the originally neutral response to LA of bugs, making LA an attractive stimulus. This behavioural change can be interpreted as appetitive conditioning. Moreover, the response to the same stimulus can be conditioned in an aversive way, by associating it with punishment. Again, *R. prolixus* was able to modify its behaviour when confronted with LA, which became this time repellent, i.e. aversive conditioning.

Insects of the control group and the L-lactic acid control group walked randomly on the locomotion compensator, confirming once more that LA is behaviourally neutral when presented alone (Barrozo and Lazzari, 2004b) as well as the absence of bias in the experimental room. When we tested bugs that were submitted to the aversive conditioning procedure, we observed a non-random walking direction, i.e. bugs avoided the LA-loaded air current. The aversive olfactory conditioning was confirmed by the results of the CS-only, US-only and unpaired US-CS groups, which presented distributions that were not significantly different from random, thus discarding the potential effects of one stimulus acting alone or the cumulative effect of unpaired US and CS.

In the first part of our study (Vinauger *et al.*, 2011a), the groups that were pre-exposed to LA (i.e. when the odour was not associated to food) showed a slight tendency to avoid the air stream loaded with LA in the olfactometer. We observed a similar effect of pre-exposition in the present study (i.e. significant circular range test, but binomial and Rayleigh tests indicating random walking). One hypothesis that could explain these observations is that pre-exposed bugs have associated LA exposure due to handling of their containers, because LA is excreted by human skin (Acree *et al.*, 1968; Bernier *et al.*, 2000). So, the experimenter himself would represent a source of LA that could have been associated with handling and manipulation. This could also explain the slight tendency of US-only group to avoid LA, when tested on the locomotion compensator. However, the appetitive conditioning group displayed a significant attraction despite handling (Vinauger

et al., 2011a), revealing that the positive reinforcement was stronger than the negative component due to handling. In other words, the perspective of completing a blood meal was stronger than the mechanical perturbation due to manipulation. Indeed, in a natural context, both types of reinforcement are present and interact.

It has often been assumed that the size of the blood meals that haematophagous insects are able to ingest is limited by the defensive behaviour of their host (Kale *et al.*, 1972; Klowden and Lea, 1979; Day and Edman, 1984; Edman and Scott, 1987), and that both factors, meal size and host defensive behaviour, affect the distribution of vectors among host populations (Kelly *et al.*, 1996; Kelly, 2001). In other words, when confronted with a host, haematophagous insects are submitted to the trade-off between the host defensive behaviour (i.e. negative reinforcement) and the value of the obtained blood meal (i.e. positive reinforcement). Given the high value of vertebrate blood and the important risks associated with its obtainment, strong selective pressures should have modelled haematophagous decision-making processes biasing their choice, favouring the exploitation of less defensive hosts. Hosts are sources of a plethora of physical and chemical stimuli that blood-sucking insects are able to perceive. Host-associated odours are mainly used by these insects to locate a potential food source but, as shown here, the response to these cues can be adjusted as a function of previous experience. This parsimonious (and plastic) use of host-derived information has an important value in terms of individual fitness by targeting blood sucking towards the less dangerous hosts. Thus, the question remains as to how positive (blood) and negative (host antiparasitic behaviour) factors influence future host selection when an insect is exposed to both types of reinforcement during the same feeding event.

An interesting finding that stood out from the analysis of walking pathways was that bugs that were conditioned to avoid the LA-loaded current did not walk exactly in the opposite direction (i.e. 180 °). Indeed, despite a significant V-test, we analysed in detail the actual trajectory and it appeared that the direction chosen by bugs was approximately 113 °. This angle is very close to that taken by triatomines walking away from moving

objects described by Lazzari and Varju (1990). These authors interpreted this particular fixation angle as a way for the triatomines to escape from a potential danger while keeping it within their visual field. This observation suggests that, independently of the sensory modality involved in the perception of a potential menace, they flee trying "to keep an eye on it". Because our experiments were conducted in darkness, we can wonder whether the bugs in the present study tried to adjust their trajectory to obtain visual contact or otherwise to optimize olfactory perception. Indeed, an airstream-borne odour coming from behind could be perturbed by the insect body before reaching the antennae.

Deviating from the exact opposite direction of the odour may thus improve olfactory detection of such odorants. Indeed, as seen in Fig. 2.5, the orientation angle during stimulation with yeast-emanating volatiles is also not exactly towards 0 °. Given that the relative position of the antennae with respect to the rest of the body in both situations, i.e. attraction and repulsion, our data are not sufficient for speculating on a relationship between walking direction and air-current perception. It should be noted, however, that no previous study has described a systematic disparity during attraction. This is a subject that deserves to be investigated in detail. The observation of deviation from the exact opposite direction raises the more general question of the application of circular statistics for the analysis of repulsion trajectories. Indeed, such tests are well adapted when dealing with oriented responses towards a stimulus, but to avoid a particular direction does not mean walking in the exact opposite direction, as revealed by our data.

Taken together, the results of the present study and those of Vinauger *et al.* (2011a) show that the same odours can acquire either a positive value when associated with blood or a negative value when associated with a mechanical shock, as bugs approach or avoid, respectively, the CS in orientation tests. So, we show that haematophagous bugs submitted to olfactory conditioning in a given context can use the learned information in a different situation. Bugs that learned an LA-blood association in the artificial feeder were afterwards attracted by this odour in the olfactometer. Conversely, bugs that learned an LA-mechanical shock association in a training jar clearly avoided this odour in the

locomotion compensator. This ability to transfer olfactory information gained in a given experimental situation to novel situations has recently been well characterized in honeybees (Carcaud *et al.*, 2009). Both dual olfactory conditioning and learning transfer between two contexts are demonstrated here for the first time in a blood-sucking insect.

2.5 Acknowledgements

The authors are indebted to F. Guerrieri and the staff members of the Neuroethology group for fruitful discussions, to C. Lahondère and F. Dupuy for critical reading of drafts and to C. Labrousse for technical support with insect rearing. This work received support from the CNRS, the University of Tours and the Agence Nationale de la Recherche (ANR-08-MIE-007, EcoEpi), France. This study was performed as part of the PhD work of C.V. at the University of Tours, under the supervision of C.R.L. and with the support of the French Ministère de la Recherche.

Conditionnement de la réponse aux signaux de l'hôte

Les punaises hématophages de l'espèce *Rhodnius prolixus* sont donc capables d'associer la même odeur de l'hôte avec la possibilité d'obtenir un repas sanguin (conditionnement appétitif) ou avec la possibilité d'être blessé ou tué par le comportement défensif de ce dernier (conditionnement aversif). Ces insectes utilisent des signaux olfactifs pour repérer leurs hôtes et sont donc capable d'apprendre à éviter un hôte dangereux et à être attiré pas un hôte sur lequel un repas a de grandes chances d'être obtenu. Mais que se passe-t-il lorsque l'insecte est confronté à plusieurs espèces d'hôtes ? La recherche d'hôtes repose-t-elle uniquement sur des comportements innés, sur l'expérience individuelle ou sur une combinaison des deux ? Afin de répondre à cette question nous avons adapté la procédure de conditionnement olfactif développée dans les deux chapitres précédents, de façon à pouvoir présenter de manière appariée, l'odeur d'un hôte (l'odeur d'un rat ou d'une caille, CS) avec la présentation d'une perturbation mécanique mimant le comportement défensif (US). Après l'entrainement, la préférence d'hôte a été testée dans un labyrinthe en Y. Tandis que les individus naïfs ne révèlent pas de préférence innée pour l'un ou l'autre des deux hôtes, les individus entrainés biaisent leur préférence en fonction de leur expérience individuelle. Les individus du groupe pour lequel l'odeur du rat a été appariée avec le choc mécanique, évitent la branche du labyrinthe délivrant l'odeur de rat et s'orientent majoritairement dans la direction de l'odeur de caille. A l'inverse, les individus du groupe pour lequel l'odeur de caille a été appariée avec le choc mécanique, ont tendance à éviter l'odeur de caille et à s'orienter dans la direction de l'odeur de rat lors du test dans l'olfactomètre. Les deux espèces d'hôtes

utilisées ici ont révélé une différence dans la difficulté à conditionner la réponse des insectes. L'histoire de vie des punaises testées, notamment les hôtes utilisés pour l'alimentation des élevages, est proposée comme explication de ces résultats asymétriques.

Les résultats obtenus sont analysés en mettant en relation l'apprentissage, le choix de l'hôte et les conséquences épidémiologiques de ces derniers.

Article soumis: Vinauger, C., Pereira, M. H. & Lazzari, C. R. (*submitted*). *Acta Tropica*

Chapitre 3

Learned Host Preference in a Chagas disease vector, *Rhodnius prolixus*

Abstract. It has been largely assumed that the individual experience of disease vector insects may affect host choice and, as a consequence, have an important influence on parasite transmission. In particular, it is speculated that vector insects should be able to learn and remember the most and/or less defensive hosts, shifting their preference accordingly. Nevertheless, despite the invested efforts in testing the capacity to learn and remember information of blood-sucking insects, only little conclusive information has been obtained hitherto. Recently, the ability of *Rhodnius prolixus* to associate a behaviourally neutral odour to the perspective of either obtaining a blood-meal or being punished has been demonstrated, the same odour becoming attractant or repellent for the bugs, respectively, according to the individual previous experience. The present work represents a step forward in the study of the cognitive abilities of Chagas disease vectors and their influence on host choice. We tested whether or not bugs bias their choice for a host based on the association of its odour with a negative experience. Our results show that whereas naïve bugs presented no preference when confronted to the odour of two different hosts, bugs previously exposed to the contingency of the odour of one host and a mechanical perturbation mimicking defensive behaviour, biased their preference towards the other host. This constitutes the first evidence of olfactory conditioning to host odours in triatomine bugs, vectors of Chagas disease and one of the few available up to date on haematophagous insects. The epidemiological significance of this finding is discussed.

Keywords: Olfactory conditioning, host-choice, Triatominae, haematophagous.

3.1 Introduction

Among the factors that could affect the transmission of parasites by blood-sucking insects, there is a remarkable lack of information regarding the cognitive abilities of dis-

ease vectors and how learning and memory might modulate their vectorial capacity. In particular, we do not know to what extent host-seeking by blood-feeding insects relies on innate behaviour, individual experience or a combination of both. Yet, a number of authors agree that investigations about the cognitive abilities of disease vectors could improve our understanding of the mechanisms underlying host preference (e.g. why some species are preferred over others), as well as the heterogeneous distribution of vectors amongst host populations (e.g. why some people are bitten more than others) (Hasibeder and Dye, 1988; Kelly and Thompson, 2000; Kelly, 2001; McCall and Kelly, 2002). Both, the spatial distribution of a population of blood-sucking insects amongst their vertebrate hosts as well as the heterogeneity in their biting behaviour have important epidemiological consequences for the transmission of important diseases, such as malaria, leishmaniasis or Chagas disease (Dye and Hasibeder, 1986; Hasibeder and Dye, 1988; Woolhouse *et al.*, 1997).

For the insect, from an evolutionary point of view, learning to recognize and remember the best hosts (e.g. less defensive and easiest to feed on) would represent an adaptive advantage. The information acquired during former foraging episodes could indicate what to avoid, as well as what to seek, during the subsequent ones (McCall and Kelly, 2002). Furthermore, the possibility to recognize the least defensive host using volatile chemical cues would provide an important benefit for the vector. Indeed, given the dispersion of chemical signals in the air, the insect could assess the risks associated with feeding on a particular type of host, from a distance where the latter is not yet a threat. The consequence would be an heterogeneous distribution of biting activity among the host population, as predicted by the ideal free distribution model (IFD) which should give place, as a consequence, to an heterogeneous distribution of parasites across different host classes and individuals (Kelly and Thompson, 2000).

Up to date, most studies examining associative learning in medically important arthropods have mostly focused on mosquitoes. Learning has been proposed to be involved in site-fidelity, oviposition preference, host preference, blood-feeding, insecticide avoiding and sugar feeding (McCall *et al.*, 2001; McCall and Eaton, 2001; Alonso *et al.*, 2003; Kaur

et al., 2003; Jhumur *et al.*, 2006; Tomberlin *et al.*, 2006). It should be noted, however, that many studies have been conducted in natural or not completely controlled conditions, others were not designed specifically for testing learning abilities and, in some cases, controlled experiments rendered negative results. So, unquestionable evidence of learning in this group of insects is very scarce (see Alonso *et al.*, 2003; Alonso and Schuck-Paim, 2006).

In triatomine bugs, vectors of Chagas disease, several attempts have been made to test their learning abilities in the past, but all of them were unsuccessful (see Aldana *et al.*, 2008 and references therein). Only recently, the ability of *Rhodnius prolixus* to modify its innate response to odours could be demonstrated (Vinauger *et al.*, 2011a, b). These studies constitute the first successful attempts to evince learning abilities in triatomines and the first to show dual olfactory conditioning (i.e. appetitive and aversive) to the same odour in haematophagous insects. In *R. prolixus*, a behaviourally neutral odour can turn into attractive or repellent, as a function of the individual experience. So, these bugs turned out to be well adapted models for the study of learning and memory in disease vectors. In addition, their physiology is well known and it is easy to have a good control on their motivation to feed, and thus to respond to hosts signals, which are essential elements for studying learning and memory.

Once the demonstration of olfactory conditioning to single isolated compounds has been done, the following step towards the understanding of triatomines learning abilities and their epidemiological relevance is to test whether or not the response of these bugs to the complete odour blend of natural hosts can be modified by learning. Thus, here we go further in analysing the way individual olfactory experience can influence subsequent host-seeking behaviour. For this, we developed an experimental paradigm allowing us to pair the odour of different hosts with a punishment simulating the host defensive behaviour and to test whether or not host-choice is influenced by the individual experience.

3.2 Material and methods

3.2.1 Insects

R. prolixus Stål were provided by the Department of Parasitology, Biological Sciences Institute (ICB) - Federal University of Minas Gerais (UFMG), where they had been maintained in colonies. The triatomines were reared under 12h:12h light:dark illumination regime, in an insectary at temperature of 28 ± 2 ℃ and relative humidity (RH) of 65 ± 10 %. They were fed weekly on chickens. Fifth-instar larvae that had just moulted were isolated in individual plastic containers and starved until the tests, 15-21 days later.

All of the assays were conducted in a room maintained at 25 ± 2 ℃, 40-60 % RH. The experiments were carried out during the first hours of the scotophase, because triatomines display a peak of activity during this period, corresponding to the moment insects leave their refuges to search for host-emitted cues in order to get a blood meal (Lazzari, 1992).

3.2.2 Experimental design

Two vertebrate host species were used: quails and rats. Hosts came from the animal house of the parasitology department. Individual hosts that have been used along experiments were randomly selected but each experimental bug was trained and tested with the odours of the same host pair.

Aversive conditioning procedure. In order to investigate whether *R. prolixus* is able to associate complex odour blends emanating from live hosts with a negative reinforcement we developed an apparatus allowing us to pair the presentation of an odour-laden air stream with a mechanical disturbance. The rationale behind this was to mimic the defensive behaviour of hosts (e.g. antiparasitic grooming).

Insects were placed in a plastic jar supplied with an air delivery system similar to that previously described (Vinauger *et al.* 2011b; Fig. 3.1), but delivering (or not) host-odour loaded air.

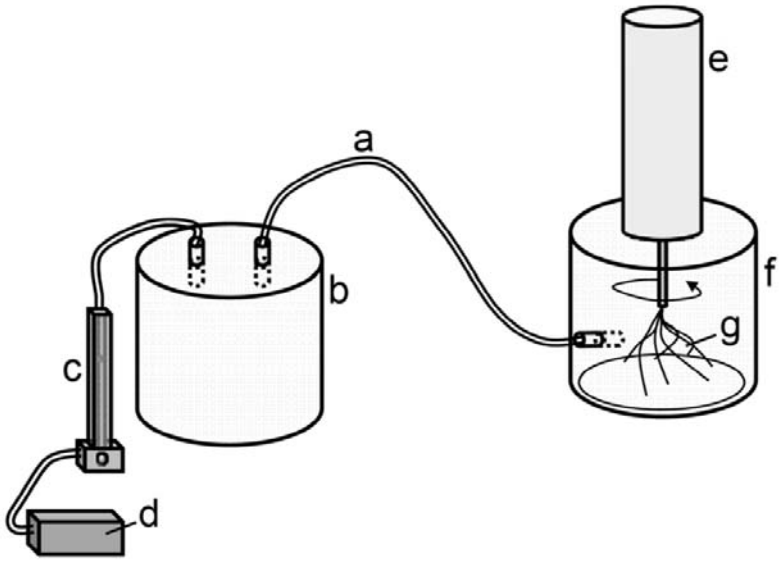

Figure 3.1: Experimental device allowing either unpaired or paired presentation of host odour (quail or rat) (CS) and a mechanical perturbation (US). a) silicone tubing; b) glass bottle containing either a rat or a quail; c) flow-meter; d) air pump; e) electric motor; f) insect container; g) paper strips (0.2-0.3 cm wide; 4 cm length).

The mechanical shock was delivered by a low speed electric motor, on which axis 15 paper strips (0.2-0.3 cm wide; 4 cm length) were fixed. This way, we were able to submit the insects to a mechanical perturbation without hurting them.

Before the training session began, insects were allowed to familiarise with the plastic jar for 2 min, without stimulation. After this time, an air current was loaded with the odour of one of the two hosts (i.e. either a quail or a rat) and it was delivered along 1 minute. During one further minute this odour-laden current was paired with the mechanical shock. Insects were submitted to 5 trials, separated by 5 minutes of inter-trial interval (ITI) (Fig. 3.2).

Testing the olfactory orientation. To compare the host preference of naïve and trained bugs to host odours, a Y-maze olfactometer was used. It consisted of a three arms enclosed maze made of Plexiglas® (Fig. 3.3). The angle between arms was 120 °. Two of the arms (i.e. choice arms) were connected to air inlets. The air streams were

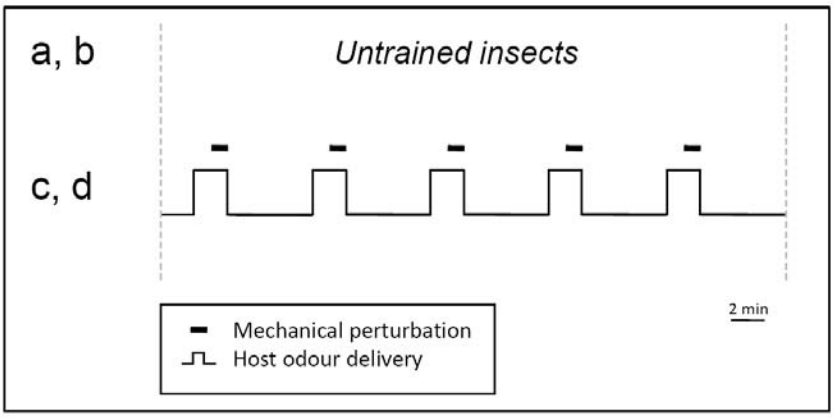

Figure 3.2: Sequence of events delivery (i.e. US, CS and ITI), during training sessions of the different experimental groups. a) neutral control group; b) spontaneous choice group; c) rat aversive conditioning group; d) quail aversive conditioning group. All training sessions had the same duration, i.e., 45 min.

generated by two independent air pumps which flow-rates were controlled by independent flow meters to be equal (4.2 $cm.sec^{-1}$, i.e. 1.18 $cm^3.sec^{-1}$). Each air current ran through a plastic jar (5 liters) containing a live host, either a quail or a rat. In order to avoid a pressure increase inside the maze, the common arm was connected to an airextracting pump which flow rate was controlled independently (13.4 $cm.sec^{-1}$, i.e. 3.8 $cm^3.sec^{-1}$). All connections were made using silicone tubing (ø= 0.6 cm).

The scene was illuminated by a red lamp, to which bugs are less sensitive than to other wavelengths (Reisenman and Lazzari, 2006). In order to avoid environmental biases, the position of the currents was randomly exchanged.

At the beginning of an experiment, one insect was placed in a familiarisation-starting chamber located at the extremity of the common arm, and closed by a nylon door. After one minute of familiarisation, the door of the starting chamber was opened. Led by its positive anemotaxis (Barrozo *et al.*, 2003), the insect walked along the common arm and at the bifurcation, it could choose to follow one of the two choice arms. We only considered the first choice made by bugs, defined as the moment when they crossed an arbitrary decision line at the entry of each choice arm. Bugs that did not choose or did not leave the starting chamber after 3 minutes were considered as not responding.

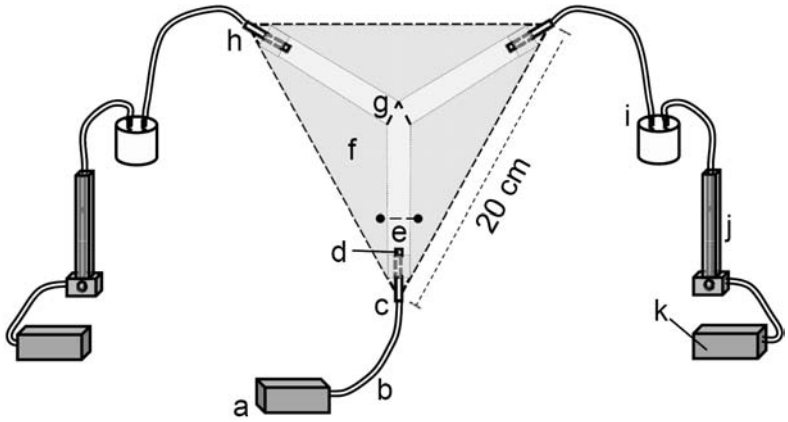

Figure 3.3: Olfactometer designed for the analysis of triatomines's olfactory orientation; a) air extracting pump; b) silicone tubing; c) glass tube; d) familiarization room; e) nylon door; f) Plexiglas® olfactometer; g) decision line; h) air inlet; i) glass bottle containing a living host (rat or quail); j) flow-meter; k) air pump.

The following groups of insects were tested:

(1) Naïve bugs of the neutral control group were tested in the olfactometer face to two identical clean air currents (n= 20).

(2) Naïve bugs of the spontaneous choice group were exposed to an air current loaded with the odour of a rat *versus* an air current loaded with the odour of a quail (n= 50).

Trained bugs of the aversive conditioning groups were submitted to the contingency between the odour of one of the two hosts (CS) and mechanical perturbation (US) as described above. Bugs were tested 15 min later in the olfactometer for their response to the two host odours (i.e. rat *versus* quail). Two groups were constituted:

(3) For the rat aversive conditioning group (n= 52), we paired the odour of the rat with the mechanical stimulation.

(4) For the quail aversive conditioning group (n=50), we paired the odour of the quail with the mechanical stimulation.

3.2.3 Data analysis

Binary data collected in the olfactometer were analysed with descriptive statistics on R software (R Development Core Team, 2010). Comparisons between groups were analysed with the Exact Binomial Test. The distribution of the insects in the olfactometer was analysed in the following way: the data distribution of each group was compared to a random distribution of 50 % on each arm of the maze. We also noted the proportion of insects that remained in the starting chamber of the maze.

3.3 Results

From their initial position in the starting chamber of the Y-maze olfactometer, insects displayed different behavioural responses according to their respective training experience. Results are depicted in Fig. 3.4.

Naïve untrained insects from the neutral control group (clean air *versus* clean air) revealed no bias in the olfactometer or in the experimental room. Indeed, 46.66 % of the insects chose one of the test arms and 53.33 % chose the other one. These results are not significantly different from a random distribution of 50 % in each arm (Binomial test: p= 0.88; n.s.).

Naïve insects of the spontaneous choice group (rat odour *versus* quail odour) did not display any preference in the olfactometer. Among them, 53.65 % chose the arm supplied with the rat odour-laden current and 46.35 % chose the other one, which air current was loaded with quail odour. These results are not significantly different from a random distribution between the arms (Binomial test: p= 0.75; n.s.), revealing no preference for one of these two host species based on their emitted odours.

The insects of the rat aversive conditioning group, which were exposed to the contingency between rat odour and a mechanical shock (Fig. 3.2), chose preferentially the arm delivering the odour of quail (74.42 %). The difference between the distribution of insects in this case and a random distribution was significantly different (Binomial test:

p= 0.0009), revealing a clearly oriented behavioural response of bugs. Conversely, only 37.21 % of the insects of the quail aversive conditioning group walked in the direction of quail odour whereas 62.79 % chose to walk towards the rat-odour laden current. However, this asymmetric orientation revealed only marginally significant (Binomial test: p= 0.06).

In all tests, most insects left the starting chamber of the olfactometer and entered into one of the decision arms. A small proportion of bugs did not respond and remained in the starting chamber (about 17.7 ± 2.1 %). This proportion was similar across the groups (Chi²: n.s.; p= 0.76), and was excluded from the statistical analysis.

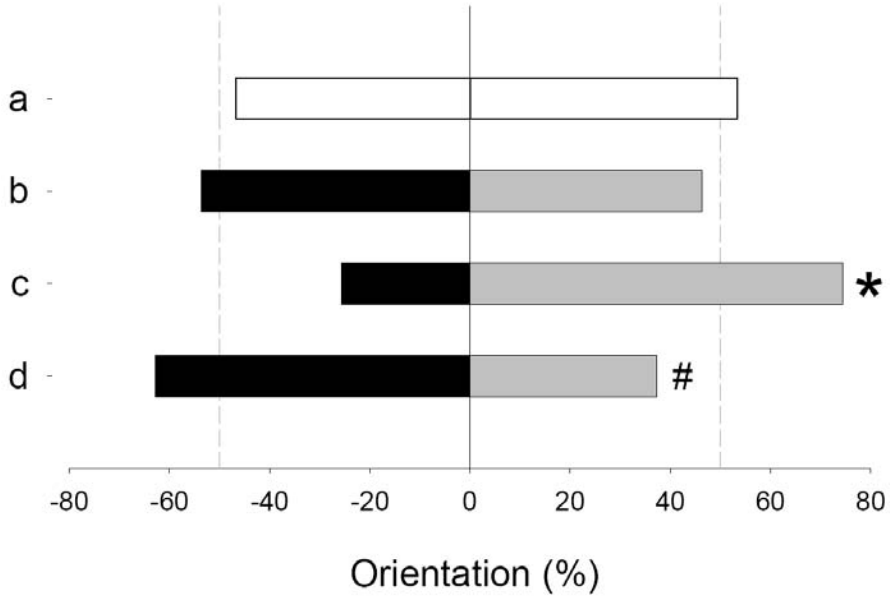

Figure 3.4: Orientation response of *R. prolixus* larvae tested in the olfactometer and confronted with two air currents either: clean (white bars) vs air loaded either with rat emitted volatiles (grey bars) or quail emitted volatiles (black bars). Orientation is represented in percentage of insects choosing each of the two test arms. Each bar represents an experimental group: "a" refers to the neutral control group (n= 20); "b" refers to the spontaneous choice group (n= 50); "c" refers to the rat aversive conditioning group (n= 52); "d" refers to the quail aversive conditioning group (n= 50). The asterisk indicates distributions that are significantly different from a random one (α= 0.05) and the # symbol indicates the marginally significant result of the Binomial test (p= 0.06).

3.4 Discussion

Learning, host choice and epidemiology. After evincing the ability of triatomines to associate single odours with positive or negative reinforcements (Vinauger *et al.*, 2011a, b), this study provides the first evidence for the possibility to manipulate experimentally the host seeking behaviour of haematophagous insects. In previous works, we tested bugs' ability to associate a single controlled volatile (L-lactic acid) with either a positive (i.e. blood reward) or a negative reinforcement (i.e. mechanical shock). Here, we analysed their ability to associate the complex blend of volatiles emanating from living hosts with a mechanical shock mimicking host defensive behaviour. Our results show that bugs did clearly bias their choice for a host, according to their previous experience. In other words, bugs are able to associate the odour of a host with the perspective of being hurt.

It should be noted that, even though the bias was induced for the odour of both hosts used in our experiments, bugs revealed a different tendency to modify the response of the insects. Whereas the odour of rats was clearly associated with a punishment, the association with that of quails was less effective, rendering only marginally significant results ($p= 0.06$). The statistical issue of this trend remained constant while increasing the number of tested insects from $n= 20$ to $n= 50$, suggesting that the difference between hosts has biological origins and is not due to the sample size. A possible explanation could be that bugs were previously fed on hens and since birds constitute the natural hosts of *R. prolixus*, it could have rendered their spontaneous choice more strongly ingrained in them. According to this hypothesis, we should have observed a preference for the quail in naïve insects when both hosts were proposed together. However, this was not the case. Our work shows that host choice is not only influenced by proximal causes, but also by previous experience, letting open the question about how innate and learned preferences interact for future research.

From a sensory ecology point of view, our results raise many questions of interest, such as: if those two host species have common components in their odours, do bugs

extract and use the differences between the odour blends or do they recognize the full odour blend as a whole? and would bugs be able to use this information to make a choice between two (or more) individuals of the same species? The available neurobiological and behavioural data do not allow answering these questions for the time being, but the possibility of conditioning bugs' responses to simple odours and to complex mixtures offers new possibilities for answering these and other relevant interrogations.

In the context of parasite transmission, the fact that vectors are able to modify their response to natural hosts' odours according to their past experience has important epidemiological consequences. As suggested by Woolhouse *et al.*, (1997), heterogeneous contact between hosts and vectors within a host population is an important determinant of the epidemiology of vector-borne diseases. Indeed, these authors identified an empirical relationship suggesting that 20 % of the host population contributes at least to 80 % of the net transmission potential, measured by the basic reproductive number, R_0. To explain this heterogeneity, it has been largely assumed and also shown experimentally, that the defensive behaviour of hosts influences the biting activity of blood-sucking insects (Edman *et al.*, 1972; Edman *et al.*, 1984; Edman and Scott, 1987). In particular, we saw here that host defensive behaviour has actually two distinct effects on insects feeding success, an immediate one by disturbing the insects while they try to feed and a postponed one by making insects associate a particular olfactory context with the possibility to be killed during the feeding process. Not only the actual antiparasitic behaviour of vertebrate hosts determines the preference of haematophagous insects, but also that the perspective to be hurt again determines host preference during subsequent blood-meals. Thus we expect experienced and non-experienced insects to distribute differentially between hosts species (inter-specific heterogeneity) and we also expect to observe a similar phenomenon among a host population (intra-specific heterogeneity). Therefore, our results add another dimension to the effect of defensive behaviour on host choice, i.e. learned host choice. This phenomenon, i.e. insects' ability to learn and remember which host was more or less defensive, will strengthen the heterogeneous distribution of vectors among host populations.

Vector cognition and disease control. To manipulate insects' behaviour is a long-held dream. In nature, insects use learned information about their environment to fine-tune their innate response behaviours to cues emanating from resources, such as food, mates or refuges. In some cases, the understanding of how insects learn to find their prey has allowed the manipulation of their behaviour in favour of human needs. For instance, some honeybees were successfully trained to associate the smell of TNT with a possible food source, in order to use the foraging properties and the high olfactory sensitivity of these insects in the context of landmines detection (Bromenshenk *et al.*, 2003; Habib, 2007). Similarly, parasitic wasps of the species *Microplitis croceipes* (Cresson) (Hymenoptera: Braconidae), have been associatively conditioned to several volatiles of interest (i.e. to detect the odours associated with explosives, food toxins, and cadavers) (Tomberlin *et al.*, 2008) and often revealed more sensitive than electronic detection methods (Rains *et al.*, 2004).

Vector learning may also have important consequences for fighting against parasite transmission. On the one hand, associative conditioning may reinforce zooprophylactic strategies, reducing fidelity to humans using infection-refractory hosts (Alexander *et al.*, 2002; McCall and Kelly, 2002). Mwandawiro *et al.* (2000) obtained experimental results suggesting that Japanese encephalitis' transmission to humans could be reduced by increasing the availability of cows (i.e. dead-end hosts for the virus) to divert mosquitoes from pigs (i.e. amplifying hosts for the virus). It is not known, however, to what extent actual defensive behaviour and learned avoiding have each contributed to this result.

On the other hand, the cognitive abilities of vectors may potentially jeopardize the employ of insecticides and repellents. It has been recently shown that insects may learn to avoid odours associated to substances having toxic effects (Ayestaran *et al.*, 2010). So, insects exposed to sub-lethal doses of insecticides could be able to avoid treated areas from distance where they can perceive olfactory cues from insecticides but without being exposed to their effects. Conversely, if bugs manage to feed on host that have been treated with repellent, would they associate the spontaneously repulsive odour with the possibility

to obtain blood? These questions should to be answered to better adapt control strategies.

Conclusions and guidelines. Our results revealed several important abilities of triatomines: 1) host choice is not only determined by the actual defensive reaction of a host, but also by their past experience; 2) bugs are able to discriminate hosts through their specific odour blends, even though they share many common components (eg, CO_2, fatty acids, etc.) and 3) they are able to recall in a context (i.e., the olfactometer) what they have learnt in a completely different one (i.e. the punishment jar).

Furthermore, this work opens new research perspectives and new potential targets for insects' control by means of behavioural manipulation In addition to exploiting triatomines activity, communication, habitat selection or dispersion, manipulating bug's host seeking behaviour could offer some unsuspected possibilities for disease control (Lazzari and Lorenzo, 2009). Nevertheless, a deep characterization of learning and memory abilities of Chagas disease vectors is first necessary and some basic questions need to be answered: How long are bugs able to retain the memorized information? Are they able to associate signals from every sensory modality with positive and negative reinforcements? Are their learning abilities modulated by endogenous and exogenous factors? Can bugs learn to recognize the odour of insecticides? to name but a few.

Finally, it is worth discussing briefly some important requirements associated to the study of learning and memory in blood-sucking insects, in order to render future work more efficient and reliable. The first element to be taken into account is the stimulus to be conditioned (CS), which should not trigger the conditioned response before training, i.e. before being paired with the unconditional stimulus. This does not mean to use a stimulus which is completely extraneous the biology of an animal, but which do not evoke the particular response that we attempt conditioning. There are many examples in the literature, even in *R. prolixus*, were stimuli to be conditioned were chosen completely outside the biology of insects and conditioning was not possible. Thus, in these studies, it is impossible to say whether bugs did not learn or did not perceive at all the proposed stimuli

(which indeed are not such for insects). In our case, we used L-lactic acid (Vinauger *et al.*, 2011a, b) and host odours (this paper) to trigger behavioural responses that were not evoked by stimuli before their association to a positive or negative reinforcements. The second is the precise control of the motivational state of insects: reproductive and nutritional status as well as age must be controlled and homogenous across individuals. The circadian clock of all the insects must be synchronized and experiments conducted always at the same daytime, which should match the temporal window when the information expected to be learnt is biologically relevant. This is because both, the sensitivity to stimuli and the learning capacities have been demonstrated to be modulated by the circadian system and vary along the day (Decker *et al.*, 2007; Bodin *et al.*, 2008). The third important aspect is a strict respect of procedures and methods imposed by this kind of studies, in particular the appropriate controls. The experiments then turn long and tedious, but this is the only way to obtain consistent and valuable results. Short-cuts have been responsible for the fail of much of the effort invested in the past (see Alonso and Schuck-Paim, 2006 for discussion).

The approach we developed was to go step by step from a highly controlled laboratory context [i.e. are triatomines able to associate an isolated odour with a reinforcement? (Vinauger *et al.*, 2011a, b)] to a more ecologically relevant one (i.e. are triatomines able to associate the complex blend of a host odour with a reinforcement?). The next steps would be to get closer and closer to the natural conditions in which insects are living, to fully understand the complexity of their cognitive abilities and their implications in their efficiency as disease vectors.

3.5 Acknowledgements

Authors are indebted to the staff members of the Department of Parasitology, Instituto de Ciencias Biologicas (ICB) - Federal University of Minas Gerais (UFMG) and of the Neuro-ethology team of our institute (IRBI – Tours, France) for fruitful discussions, in particular to C. Lahondère for critical reading of drafts. Authors are also thankful to César Nonato de Oliveira for his technical support and particularly with insects rearing.

This work received support from the CNRS, the Agence Nationale de la Recherche (grant ANR-08-MIE-007, EcoEpi), the University of Tours, the doctoral school Sciences Santé et Technologies, Tours - France. This study was performed as part of the PhD work of C.V. at the University of Tours, under the supervision of C.L. and with the support of the French Ministère de la Recherche.

Deuxième partie

Conditionnement de la réponse d'extension du proboscis (PER) chez un insecte hématophage

Après avoir démontré la possibilité de conditionner la réponse des triatomines aux signaux olfactifs émis par les hôtes et perçus à longue distance, nous nous sommes intéressés à la chaleur, signal utilisé à proximité de l'hôte pour guider et déclencher le comportement de piqûre (Flores et Lazzari, 1996 ; Ferreira *et al.*, 2007). La chaleur étant le seul signal nécessaire et suffisant pour provoquer la réponse d'extension du proboscis (PER) des triatomines, nous avons adapté le paradigme de conditionnement de la PER développé chez les modèles classiques (i.e. drosophile et abeille), en utilisant le sens thermique comme modalité sensorielle. L'objectif de ce chapitre est de mettre en place un paradigme expérimental, efficace et reproductible, permettant l'analyse détaillée des capacités d'apprentissage d'un insecte hématophage. Nous nous sommes donc intéressés dans un premier temps à la mise en évidence d'une forme d'apprentissage non-associatif, l'habituation. En soumettant les insectes à des stimulations appétitives répétées (i.e. un objet à 35°C) nous avons pu observer une diminution progressive de la réponse à la chaleur, allant jusqu'à la disparition totale de la réponse (i.e. absence de réponse pendant plusieurs essais consécutifs). Une expérience de déshabituation a confirmé une réelle habituation de la réponse, en écartant les effets de la fatigue motrice et d'une éventuelle adaptation sensorielle. Nous avons également pu mettre en évidence la formation d'une trace mnésique à la suite de la procédure d'habituation. Ces expériences ont de plus révélé un effet du contexte expérimental sur l'apprentissage.

Dans un second temps nous avons mis en place un protocole de conditionnement aversif de la PER. Cette procédure nous a permis d'estimer la durée de rétention maximale de l'information mémorisée, c'est la dire la durée de persistance de la marque mnésique. Si 72h après l'entrainement, les individus montrent des performances significativement meilleures que des individus naïfs, l'effet de l'entrainement n'est plus visible 96h post apprentissage.

Ces résultats démontrent qu'il est possible de placer des insectes hématophages dans un cadre expérimental permettant d'analyser et caractériser les processus d'apprentissage et de mémorisation. Le paradigme proposé ici devrait permettre d'approfondir la compréhension de ces processus et l'analyse des mécanismes moléculaires et neurobiologiques sous-jacents.

Article en préparation: Vinauger, C., Lallement, H. & Lazzari, C. R. (*in prep.*

Chapitre 4

The Proboscis Extension Response as a paradigm for the study of learning and memory in disease vector insects

Abstract. Learning abilities are supposed to play a key role in parasite transmission by disease vectors. However, the experimental evidence is scarce due to experimental constrains associated with haematophagy. Most of the conclusive studies were conducted under natural or partially control conditions and thus do not provide any standardized methodology that would allow answering questions regarding the physiological, neurobiological or molecular mechanisms underlying learning and memory. Applying a conditioning procedure that has been widely validated in drosophilae or honeybees, we demonstrated that the proboscis extension response (PER) of an haematophagous insects could be modulated by non-associative (habituation) and associative (aversive conditioning) learning forms. In the first part of this work, the PER was habituated and dishabituated, demonstrating true central processes and discarding motor fatigue or sensory adaptation. In the second part, bugs that were submitted to aversive conditioning, were able to use their past experience in order to adapt their PER when tested up to 72h post-training. Context effect and molecular basis of memory were also investigated in this study. This constitutes the first demonstration of PER habituation and conditioning in a blood-sucking insect and provides a reproducible experimental paradigm allowing the in depth study of learning and memory of disease vectors.

Key words: PER, triatomines, habituation, dishabituation, aversive conditionning, memory retention.

4.1 Introduction

In some insect species, feeding on liquid food, mouthparts have evolved toward a tubular feeding and sucking organ, known as the proboscis, latinisation of the greek *proboskis*, which comes from pro "forth, forward, before" and *bosko*, "to feed, to nourish". To obtain their food, these insects extend their proboscis in a stereotyped behaviour, referred to as the proboscis extension response or PER. This behavioural response to food signals has been widely used in taste behaviour assays (Frings, 1941; 1944; Hayes and Liu, 1947; Grabowski and Dethier, 1954) and turned out to be a key paradigm in the study of the behavioural and cognitive plasticity of insects (Takeda, 1961; Bitterman *et al.*, 1983).

As true as vertebrate physiologists have their white laboratory rats, invertebrate neurobiologists focused their studies on few classical insect models. Among them, drosophilas and honeybees learning abilities were intensively investigated. Adapted from studies on the blow fly *Cynomyopsis cadaverina* (Frings, 1941) and used for the first time in hymenopterans by Frings (1944), PER conditioning in honeybees was employed by Kuwabara (1957), Takeda (1961), Bitterman *et al.* (1983) and many others thereafter, to study the behavioural, molecular and neurobiological processes underlying learning and memory in insects.

Major advances in this field of knowledge were done in the honeybee *Apis mellifera* by means of classical appetitive conditioning procedures (Bitterman *et al.*, 1983; Menzel and Muller, 1996; Erber *et al.*, 1997; de Brito Sanchez *et al.*, 2005) or more complex conditioning forms (e.g. second order conditioning, differential conditioning, etc) (Deisig *et al.*, 2001; Giurfa and Malun, 2004; Châline *et al.*, 2005). In dipterans, *Drosophila melanogaster* revealed as a excellent model to go deeper in the understanding of learning and memory, applying PER aversive conditioning procedures (Vaysse and Medioni, 1976; De Jianne *et al.*, 1985) or olfactory conditioning (Holliday and Hirsch, 1986; Fresquet, 1999; Chabaud *et al.*, 2006).

In haematophagous insects, blood feeding goes through getting access to fluid that is

hidden under hosts' skin. To do so, blood-sucking insects have to locate their prey and, when close enough, extend their proboscis to pierce through the skin. These two steps are achieved thanks to behavioural response to olfactory and thermal signals (Lehane, 2005). Thus, to be able to learn to recognize the less defensive hosts, i.e. the easiest to feed on, seems to be adaptive and one would expect to observe well developed cognitive abilities in these insects as well (McCall and Kelly, 2002; Alonso *et al.*, 2003). Furthermore, it has been largely admitted that learning and memory are two factors that could explain the heterogeneous distribution of vectors among host species and populations (Kelly and Thompson, 2000; Kelly, 2001; McCall and Kelly, 2002). In terms of epidemiology, such heterogeneities in the biting strategies of insects mean heterogeneities in the transmission of infections agents. Woolhouse *et al.* (1997) suggested that 20 % of the host population contributes 80 % of the net transmission potential. In other words, learning and memory are two factors participating to the creation of extreme transmission "hot spots" and "cold spots" (Kelly, 2001).

However, in spite of the intense research effort invested so far, only few studies have given clear experimental demonstration of learning and memory in haematophagous insects (Alonso and Schuck-Paim, 2006). Most of the conclusive studies available were conducted under natural or partially control conditions (Mwandawiro *et al.*, 2000; McCall and Eaton, 2001; Bouyer *et al.*, 2007) and thus do not allow the employ of a standardized and easy to use methodology that would enable researchers to dive deeper in the understanding of the underlying mechanisms.

In triatomine bugs, vectors of the Chagas disease, the responses of *Rhodnius prolixus* to a single olfactory stimulus can be modified by either appetitive or aversive conditioning (Vinauger *et al.*, 2011a, b). Similarly, their host preference has been demonstrated to be under the influence of previous individual experience (Vinauger *et al.*, submitted). However, those studies aimed at demonstrating the ability of these insects to learn information about their hosts. They were thus designed to place the insects in an experimental context that was as favourable as possible for the observation of learning abilities, but the

experimental paradigms developed in these works were not designed for allowing a deeper analysis of the mechanisms underlying learning and memory processes.

R. prolixus revealed as an adequate model for setting up such an experimental paradigm. Indeed, its biology and physiology have been well described and allow the experimenters to adapt PER conditioning methodologies developed in classical insect models. For instance, heat is a necessary but sufficient signal to trigger the PER (Flores and Lazzari, 1996) but also a signal that can be used in an aversive context (Okasha; 1968a, b).Thus, the goal of the present work was to set up and validate a classic experimental paradigm, the PER, to apply it to the study of learning and memory in haematophagous insects, using their thermal sensitivity.

4.2 Material and Methods

4.2.1 Insects

Fifth-instar larvae of *Rhodnius prolixus* were used throughout the experiments. Bugs were reared in the laboratory under a 12h:12h light:dark illumination regime, at 27 ± 2°C and 60-70 % relative humidity (RH). Insects were fed weekly on sheep heparinised blood, using an artificial feeder (Núñez and Lazzari, 1990). Fifth-instar larvae that had just moulted were isolated in individual plastic containers and starved until being tested, 15 days after their moult.

4.2.2 Experimental apparatus

Insects were tethered by their dorsal thorax to a stiff steel wire, using double-sided adhesive tape, in an experimental room which temperature was kept at 25 ± 2°C (Fig. 4.1). A Styrofoam ball was placed between their legs in order to provide tarsal contact and, this way, to reduce unnecessary stress. A Peltier element, representing an accurate and controllable heat source, was placed in front of animals, at a distance from which they could

touch the surface while extending their proboscis. This element allowed rapid temperature changes of the surface that was presented to the insects and, this way, we could display an appetitive heat source (30-35℃) or apply a negative reinforcement (50℃) (see below). The efficiency of the Peltier element was improved by a water cooling device placed on the back of the apparatus to cool down the dissipated heat. Thus, the element temperature could switch from 25℃ to 35℃ and from 35℃to 50℃, according to experiments, very quickly (Δ 25℃ in less than 1 sec). A thermal sensor was placed in contact with the element Peltier and used to control the temperature of the device.

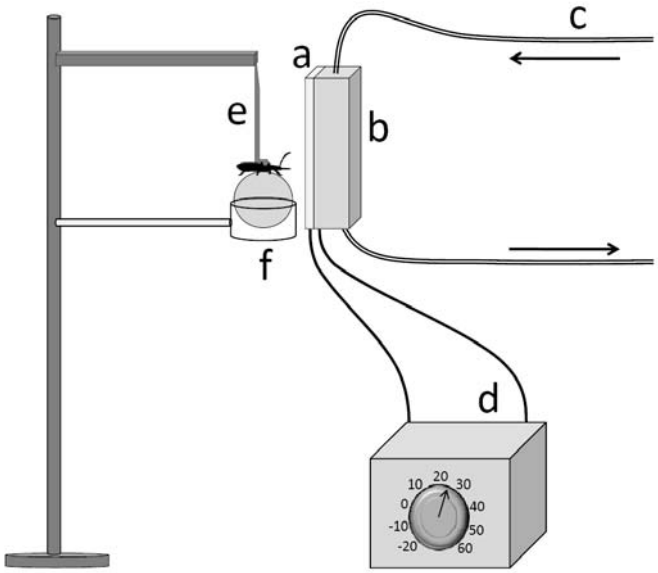

Figure 4.1: Experimental device used for training the PER of *R. prolixus*. It allows the delivery of thermal stimulation. a, Peltier element; b, Aluminium heat dissipator; c, Enclosed water based cooling; d, Thermostat; e, steel wire allowing the fication of an insect; f, Styrofoam sphere (1 cm diameter).

The assays were monitored with the aid of an infrared-sensitive camera provided with an array of infrared LEDs (emission 900 nm). This light illuminated the scene without being perceived by the bugs (Reisenman *et al.*, 1998) and allowed us to observe proboscis movements with more details.

Two distinct series of experiments were conducted in order to study two different forms

of learning, a non-associative one, habituation and an associative one, aversive conditioning. In the first series, the habituation of the PER along successive stimulation at 35℃, was tested. In the second series, we tested whether or not bugs are able to associate their response to an appetitive thermal stimulus (35℃), i.e. the PER, with the trigger of an aversive heat shock (50℃). In both experiments, the environmental temperature was chosen according to the relative knowledge on thermal preferences and tolerances of these insects (Okasha, 1968a, b; Schilman and Lazzari, 2004). The appetitive one, of 35℃, corresponds to host skin surface temperature and the aversive one, of 50℃, is not lethal and represents warm objects different from hosts that the animal may encounter in its natural environment (e.g. cooking elements in host's houses, stones exposed to sunlight, etc.).

At the beginning of each experiment, bugs were placed individually in the device and were accorded a 30s period in order to familiarize with the experimental situation. During this period, the temperature of the Peltier element was fixed at 25℃, corresponding to the room temperature.

Then, they were submitted to several successive trials, separated by 50 sec inter trial intervals. During these intervals, the Peltier temperature was maintained at 25℃.

During trials, the occurrence or absence of the PER at 35℃ was noted, and the percentage of insects responding to heat stimulation was calculated. Each individual was repeatedly submitted to trials until complete disappearance of the response, i.e. three successive trials without any answer.

All insects that did not respond to heat during the two first trials were considered as not motivated and were discarded from analysis. A proboscis extension response was counted when the proboscis was fully extended, that means when displaying an angle of 180 ° from its initial position.With the aim of exploring the utility of the PER as learning bioassay, we conducted different series of experiments for testing: habituation, dishabituation, the effect of context and the aversive conditioning of the PER. This last paradigm was used for measuring the persistence of the mnesic trace.

4.2.3 Habituation

After the 30 sec of familiarization time, bugs are submitted to successive presentations of 10 sec appetitive stimulation (i.e. Peltier at 35℃). Three experiments were carried out (Fig. 4.2):

1a) PER habituation. Each individual was placed in the experimental device while the Peltier was at room temperature (25℃). After the familiarization period, the bug was submitted to successive trials during which the temperature of the Peltier was increased to 35℃ during 10 sec. Trials were separated by a 50s inter trial interval (ITI) during which the Peltier was brought back to room temperature. Insects remained in the device until the end of the session, i.e. until complete disappearance of the response to the appetitive stimulus (n= 16).

1b) PER dishabituation. In order to shed more light on the mechanisms underlying the disappearance of the response to heat, a dishabituation experiment was conducted (n= 10). The first 12 trials of this experiment were similar to the habituation procedure (i.e. room temperature at 25℃ and a stimulation at 35℃ during 10 sec see exp. 1a), then, from the 13^{th} trial, properties of both the thermal stimulus and the temperature of the Peltier during ITIs were modified. During the dishabituation phase, the stimulation and the ITI temperatures were lowered to 20℃ and 30℃ respectively. No changes were made in either the durations of stimulation and ITI.

The beginning of the dishabituation period was determined according to the mean number of trials that were necessary to observe an habituation of the PER during the first experiment. The habituation and dishabituation phases of the experiment were only separated by the duration of an ITI (i.e. 50 sec). The aim of this experiment was to discriminate whether the reduction of response frequencies along trials was due to sensory adaptation or motor fatigue (peripheral processes) or due to true non-associative learning (central process).

1c) Retention and context effect. To test whether or not habituation gives place to a mnesic process, we tested the influence of a first habituation session (training session) on the performances during a second habituation session (test session) performed 1 hour later. Procedures and temperatures were the same as in exp. 1a. Between training and test sessions, insects were placed in individual plastic jars and brought back to the rearing room (n= 10).

Performances were compared to control groups that were untrained during the first session. Furthermore, in order to discard any context effect, control individuals were placed in the experimental device during the mean duration of the training session of the associated test group. During this first session, the Peltier element was kept at room temperature (25°C).

The influence of the experimental context on memory retention was tested in this experiment. For this, two groups of insects were constituted, a trained one (n= 11) and a control one (n= 10). Procedures were identical to the retention experiment except that the first session of the control group was not conducted in the experimental device but in a completely different context. In addition, in order to be able to discard any effect due to manipulation of insects by the experimenter, control bugs were submitted to the same handling procedure as the trained ones. Indeed, they were tethered by their dorsal thorax to a stiff steel wire, but instead of being placed in the experimental device afterward, they were placed under darkness in an opaque plastic jar (5 cm height 3 cm diameter), with a small paper piece between their legs, thus providing a tarsal contact and reducing stress. The duration of this first session was set as the mean time necessary to observe a complete habituation of PER in the first session of the trained group. In this experiment first and second sessions were separated by 1h for both trained and control bugs.

4.2.4 Aversive conditioning

We carried out aversive conditioning experiments, testing the ability of *R. prolixus* to stop its proboscis extension response (PER) face to a thermal stimulus (35°C), when paired

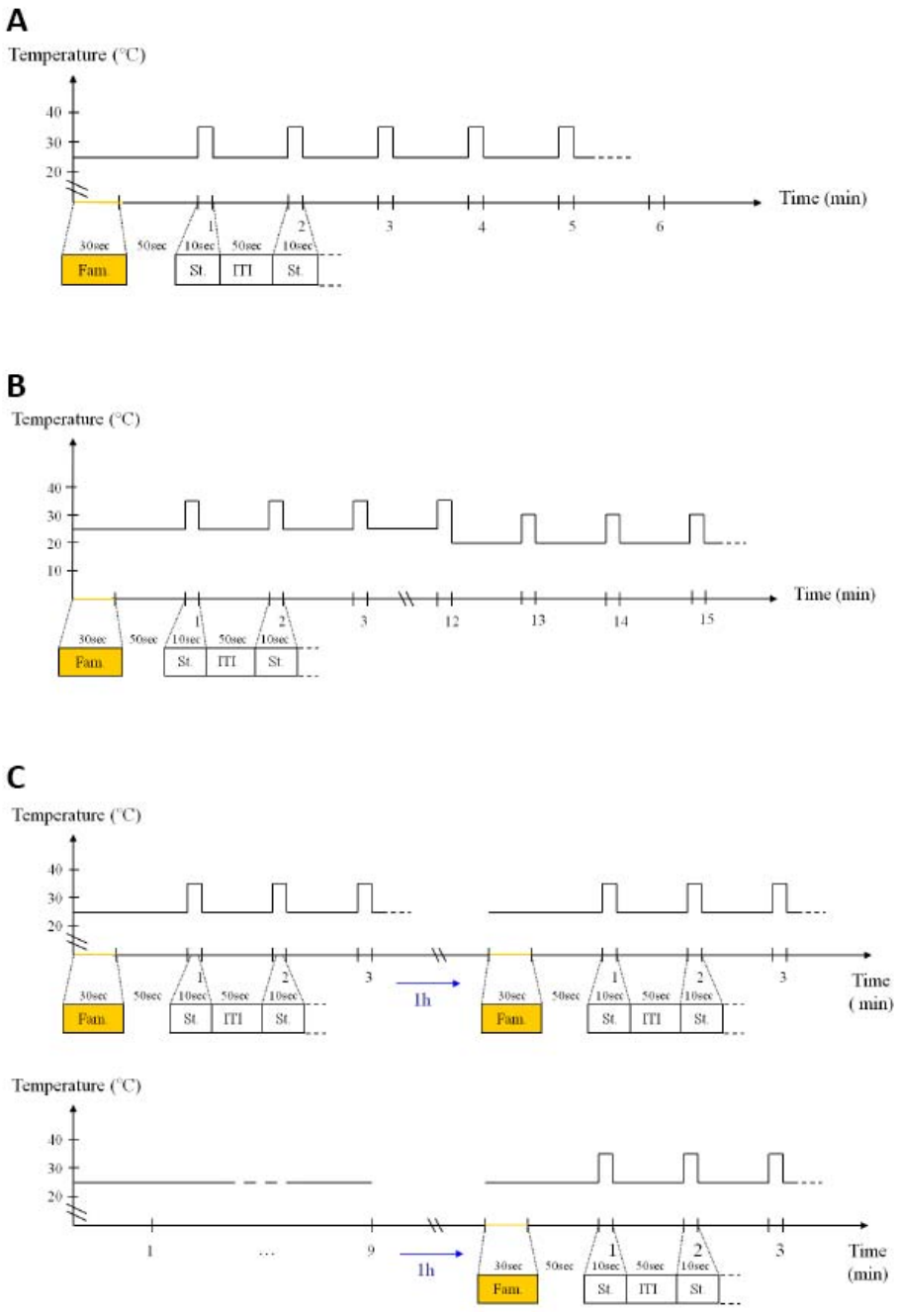

Figure 4.2: Sequence of event delivery (i.e. appetitive thermal stimulation and inter-trial interval) during training sessions of the different experimental groups: (A) PER habituation; (B) PER dishabituation; (C) Retention and Context effect.

with a thermal shock (50°C). Three experiments were carried out (Fig. 4.3):

2a) Aversive conditioning of PER. Bugs of this experimental group were submitted to repeated trials, after a 30 sec familiarisation period. One trial being defined as follow:

(a) Appetitive stimulation (35°C) during 10 sec.

(b) In case of PER, i.e. insects responding to the thermal stimulation, a heat shock was delivered to the extended proboscis at the end of the 10s period, by increasing the temperature of the Peltier to 50°C. If no PER was displayed, at the end of the 10s stimulation, insects did not received any reinforcement.

(c) Inter-trial interval of 50s.

Insects reacted to the heat shock by retracting their proboscis and by displaying stress-associated behaviours (e.g. rapid movements of legs, head and antennae). Once the proboscis was folded again the temperature of the Peltier was reduced to 25°C. Results were compared to those obtained in the habituation experiment (exp. 1a) in order to assess the influence of a negative reinforcement on learning performances and, in particular, on acquisition speed.

2b) Retention experiments. Bugs underwent two sessions, a training one and a test one, following the same procedure as exp. 2a. Four trained groups were constituted in order to test: 1) whether or not training influences the performance during a subsequent test session and 2) the maximal retention time length. Thus, for each group, training and test sessions were separated a different time interval: 1h (n= 12), 24h (n= 14), 72h (n= 12) and 96h (n= 14).

A control group was run in parallel to each experiment, which individuals were handled in an identical manner, but not trained during the first session, i.e. they were placed in the set up and exposed to the Peltier at a constant temperature of 25°C, during the mean time of a training session (determined as the time necessary to observe complete disappearance

of the PER in the respective trained groups). Insects of the control groups were then tested in a second session, as the associated trained groups.

4.2.5 Data analysis

Learning performance of individual insects were quantified by determining the number of trials required to observe the disappearance of the PER along three consecutive trials (Braun and Bicker, 1992). A mean performance was then calculated for each group. Provided that not all the samples passed the normality test, non-parametric statistics was used throughout. Wilcoxon signed-rank tests for paired data were used to compare performances between training and test sessions of the same group and comparison between the performances of trained and untrained control groups were made by using Mann-Whitney test for independent samples (α= 0.05). All statistical tests were computed using R software (R Development Core Team, 2010).

4.3 Results

4.3.1 Habituation

1a) PER habituation. The habituation dynamic of PER is represented in Fig. 4.4a. With the repetition of thermal stimulation, the percentage of bugs extending their proboscis in response to the stimulus progressively decreases along trials, down to zero. A mean of 25.6 $\pm$ 4.7 trials were necessary to observe a complete disappearance of PER.

1b) PER dishabituation. To discard the influence of peripheral processes such as sensory or motor adaptation, we tested whether a change in the experimental parameters (i.e. stimulus and ITI temperatures) could restore the initial reactivity to appetitive thermal stimulation.

In a first time, insects of this experimental group displayed a typical habituation response during the first twelve trials (from 80 % of PER at the first trial to 30 % at the

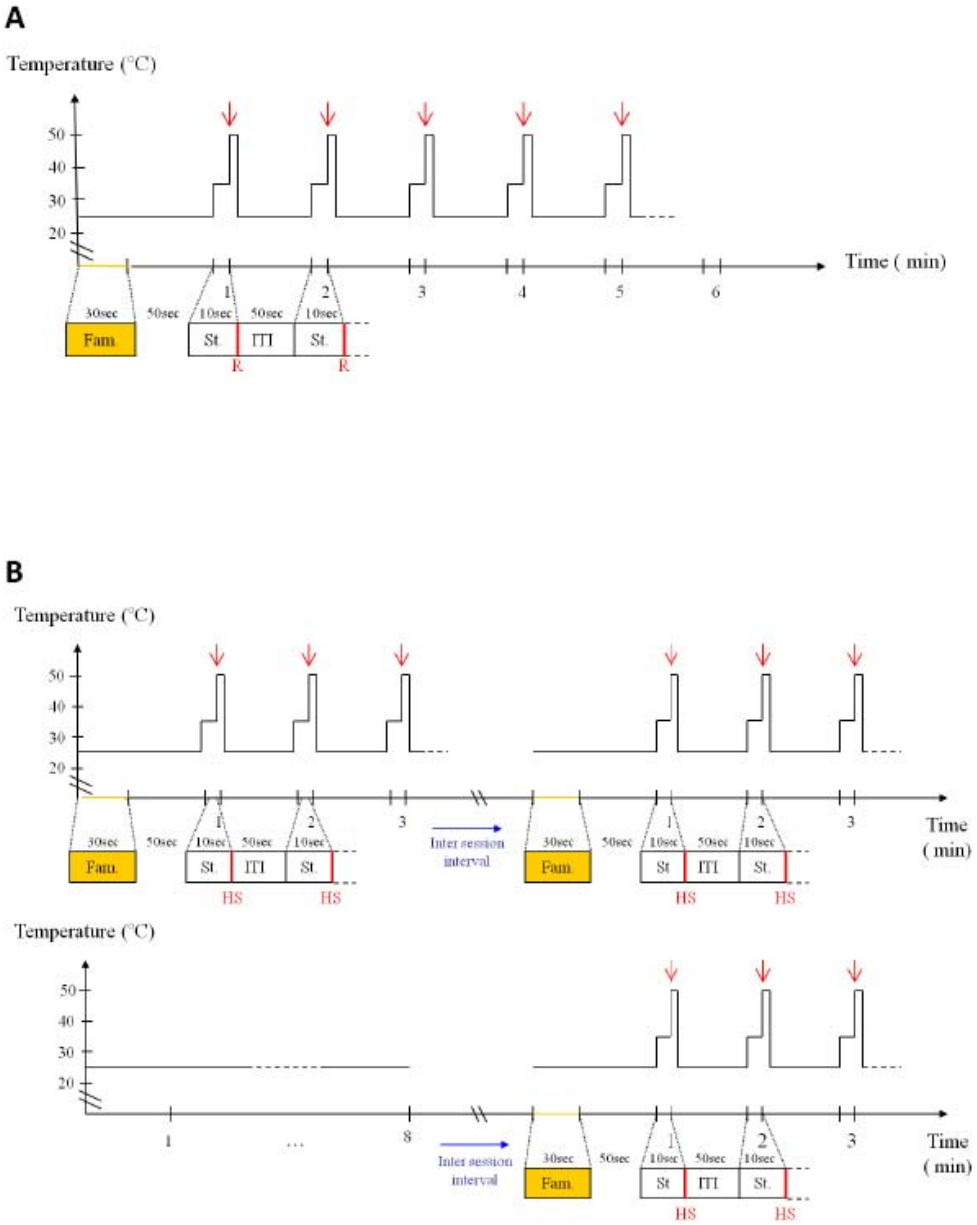

Figure 4.3: Sequence of event delivery (i.e. appetitive thermal stimulation and inter-trial interval) during training sessions of the different experimental groups: (A) Aversive conditioning of PER ; (B) Retention Experiments.

12^{th} Fig. 4.4b). Then, temperatures of stimulus and ITI were reduced from 35 to 30°C and from 25 to 20°C respectively. We then observed that the percentage of responses increased to 50 % at the 13^{th} trial and 80 % at the 14^{th} one, i.e. the same level of responsiveness as at the beginning of the habituation phase.

It worth mentioning that this increase was not instantaneous since it required two trials after temperature changes before observing levels as high as at the beginning of the session. In addition, once the maximal percentage of PER was reached, we observed an habituation dynamic, similar to that observed during the first phase of the experiment. These results demonstrate that the habituated PER can be dishabituated by modifying the parameters of the stimulus and the ITI. Furthermore, they additionally demonstrate that the decrease in the response does rely on learning abilities rather than on peripheral mechanisms.

1c) Retention and context effect. Results are depicted in Fig. 4.5a. In the 1h retention test, trained bugs required less trials to stop responding to the stimulus during the test session than during the training one (S1 : 14.3 ± 3 trials; S2 : 3.5 ± 1 trials; Wilcoxon test: p= 0.002). In addition, the number of trials required to observe a complete habituation was significantly different between the control group and the test session of the trained group (Mann-Whitney text: p= 0.019). The number of trials required being superior in the control group (8.8 ± 2.13 trials).

It worth mentioning that the performance of the control group was quite different, although not significant (Mann-Whitney test: p= 0.074), from the performance of trained bugs during their training session.

To go deeper in the understanding of the potential context effect we performed a similar experiment but, this time, exposing control bugs to a context that was different from the experimental one. Results are depicted in Fig. 4.5b.

Trained bugs displayed similar results and stopped responding sooner during the test session than during training (S1: 8.9 ± 1.6 trials; S2: 3.5 ± 1.1 trials; Wilcoxon test: p= 0.006). However, no difference could be observed between performances of the control

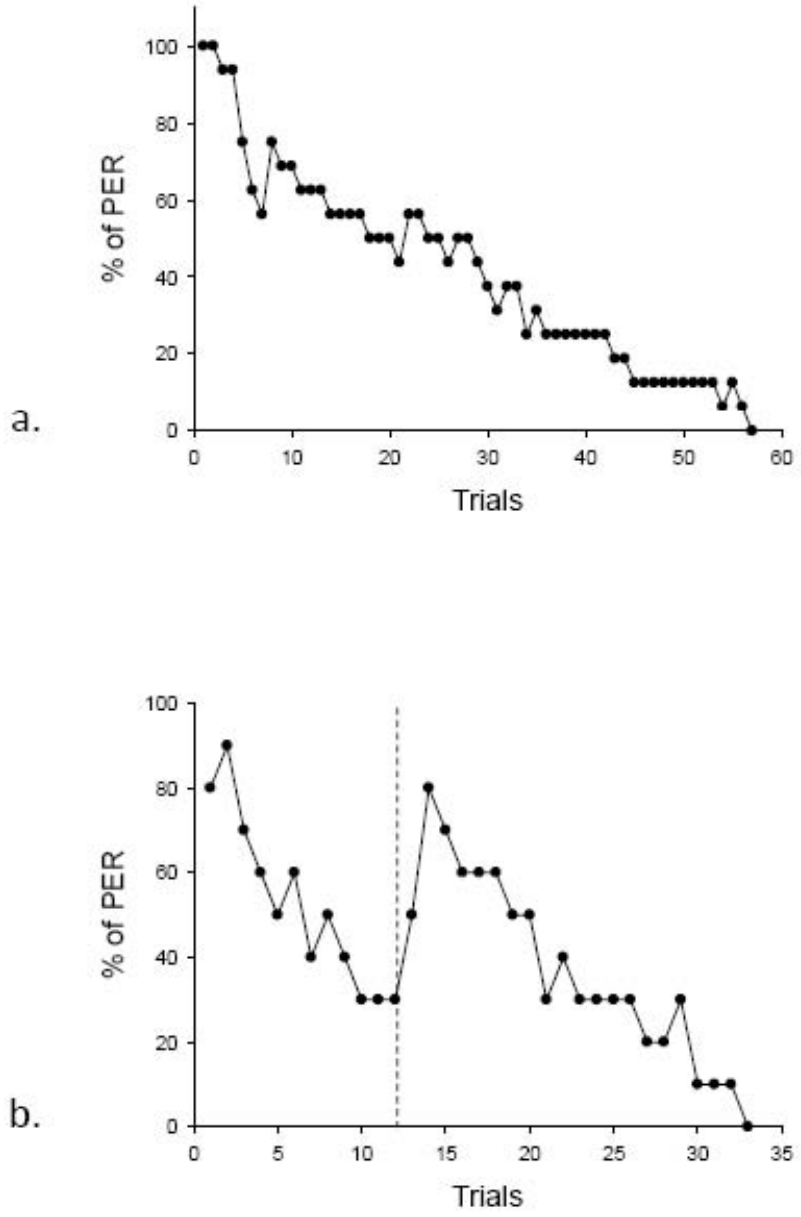

Figure 4.4: Percentage of *Rhodnius prolixus* larvae responding to the appetitive stimultation (35℃) along trials. (a) Habituation of PER; (b) Dishabituation of PER.

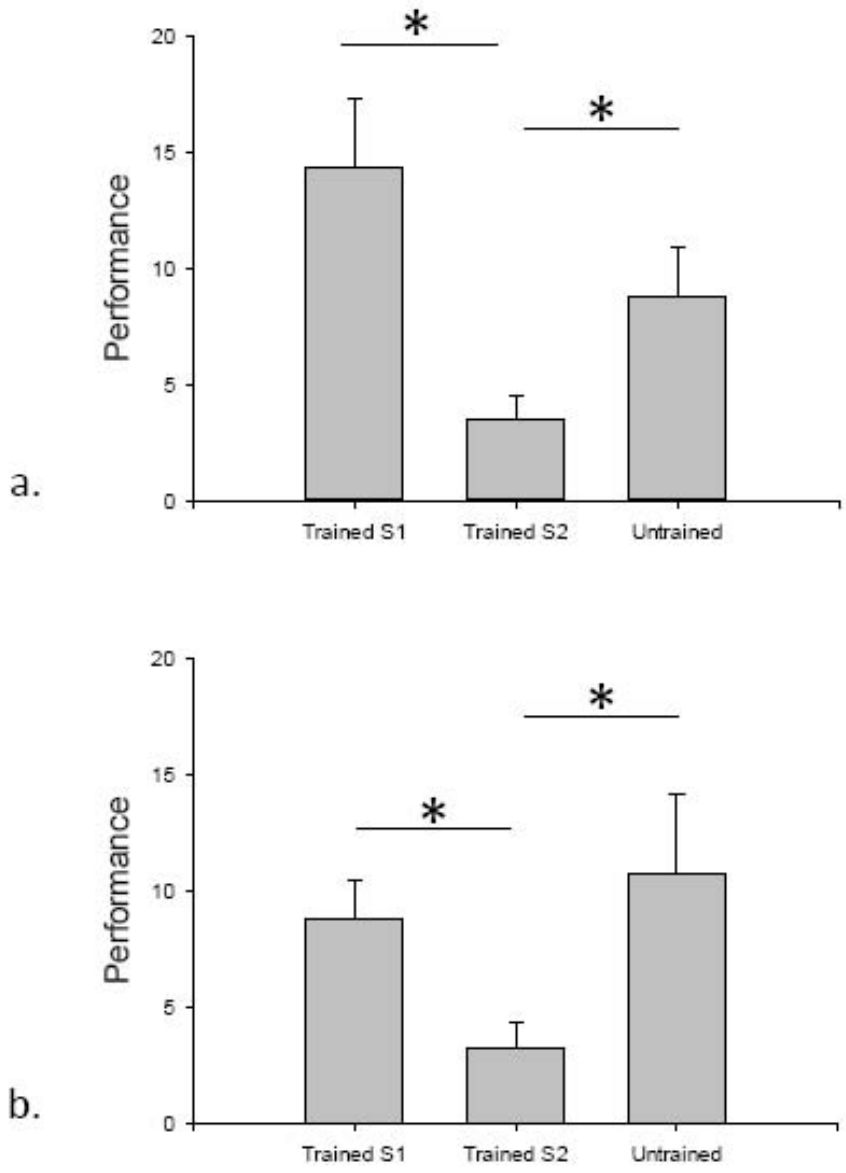

Figure 4.5: Performances of *Rhodnius prolixus* larvae, representing the mean number of trials that were necessary to observe a complete disappearance of the response. Each bar represents either a trained group during its first (S1) or second session (S2) or the associated control group (Untrained). Asterisks indicate significant differences ($p<0.05$).

group (10.7 ± 3.5 trials) and the first session of the trained group (Mann-Whitney test: $p = 0.40$). Thus, 1h after having been exposed to a context different from the experimental one, learning performances were similar to naïve bugs, i.e. to those observed during the first (training) session of the trained group. Furthermore, since insects of both groups (trained and control) were submitted to the same manipulations, and taking into account the fact that performances were significantly different between control and tested bugs (Mann-Whitney test: $p = 0.006$), we can discard a potential effect of manipulation on learning performances.

4.3.2 Aversive conditioning

2a) Aversive conditioning of PER. When the response to a thermal stimulus was negatively reinforced with the delivery of a heat shock, bugs required fewer trials to stop responding (Fig. 4.6).

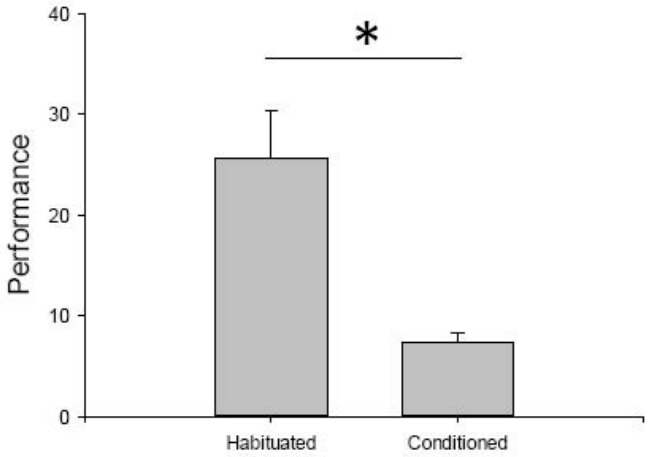

Figure 4.6: Performances of *Rhodnius prolixus* larvae, (defined as the mean number of trials that were necessary to observe a complete disappearance of the response) submitted to habituation and aversive conditioning procedures. Asterisk indicates significant differences ($p<0.05$).

In comparison to the non-reinforced group, i.e. habituated group, we observe a more rapid decrease in the percentage of PER per trials. In both groups 100 % of bugs extend

their proboscis during the first trial. However, while it required 26 trials to observe a 50 % reduction of the response in the habituated group, this decrease was observed at the 5^{th} trial in the reinforced group.

Similarly, the mean number of trials that were necessary to observe a complete disappearance of PER, was significantly lower in the reinforced group (7.3 ± 0.9 trials against 25.6 ± 4.7 in the habituated group; Mann-Whitney test: $p = 0.004$). These results suggest that triatomine bugs are able to associate their response with a negative reinforcement and adapt, by stopping responding in order to avoid heat shocks.

2b) Retention experiments. The ability of bugs to store, retain and retrieve the learned information was explored in this experiment.

The first retention duration was 1h post training (Fig. 4.7a). The percentage of bugs responding at the first trial was higher during the training session (100 %) than during the test session (33 %). The untrained group seemed to display an intermediate learning profile, with 80 % of PER during the first trial. Regarding the mean number of trials that were necessary to observe a complete disappearance of PER, we observed that bugs stopped responding sooner in the test than in the training session (training: 7.9 ± 1.1 trials; test: 3.3 ± 0.8 trials; Wilcoxon test: $p = 0.004$). Furthermore the significant differences between the performance of untrained and trained insects during their test session (Mann-Whitney test; $p = 0.009$), discarded potential effect of the context in this situation. In addition, the difference in performances between naive insects and bugs pre-exposed to the context was not significant (Mann-Whitney test; $p = 0.076$).

When, the interval between training and testing was increased to 24h, we still observed a significant effect of training on the performances during the test session (training: 4.2 ± 0.5 trials; test: 2.9 ± 0.3 trials; Wilcoxon test: $p = 0.\ 0054$; Fig. 4.7b). Performance during test session was also significantly better (i.e. required fewer trials) than the performance of untrained bugs (4.8 ± 0,6 trials; Mann-Whitney: $p = 0.011$). However, this time, no significant difference was observed between naïve insects during their training session and

bugs that were pre-exposed to the experimental context (Mann-Whitney test: $p= 0.424$), discarding any effect of context in this retention test.

Similar results were obtained when the retention was tested 72h after training (Fig. 4.7c). Performances were improved by learning since insects required fewer trials during the test session than during the training one (training: 6 ± 1.6 trials; test: 2.9 ± 0.8 trials; Wilcoxon test: $p= 0.019$) and fewer trials than untrained bugs (untrained: 6.9 ± 1.6 trials, Mann-Whitney test: $p= 0.006$). No significant difference was observed between performances of naïve insects (training session) and bugs pre-exposed to the experimental context (Mann-Whitney test: $p= 0.53$), discarding context effect in this retention test.

Conversely, when tested 96h after training, bugs did not stop responding sooner than during training (training 5.5 ± 1.1 trials; test: 4.3 ± 1.1 trials; Wilcoxon test: $p= 0.078$; Fig. 4.7d). In other words, no effect of training was observed on the performances of bugs, 96h post-learning.

4.4 Discussion

This study shows that methodologies developed on classical models for the study of learning and memory can be adapted to haematophagous insects. Indeed, the PER of triatomine bugs revealed as an appropriate behavioural response for the study of learning mechanisms (both, non-associative and associative), as well as to go deeper into the neurobiological basis of memory retention. It should be noted, however, that this adaptation to blood-feeding models requires overcoming some major constrains associated with haematophagy. For instance, in nectar feeding insects, PER can be elicited via the direct contact of sugar solutions (Unconditional stimulus, US) on taste receptors. It is then relatively easy to pair an odour or other stimulus (Conditional stimulus, CS) with the US and test whether or not the insect has associated both stimuli and extends its proboscis to the only delivery of the CS. In haematophagous bugs, PER is only triggered by appetitive thermal stimulation (US). In other words, heat is the only stimulus both, necessary and

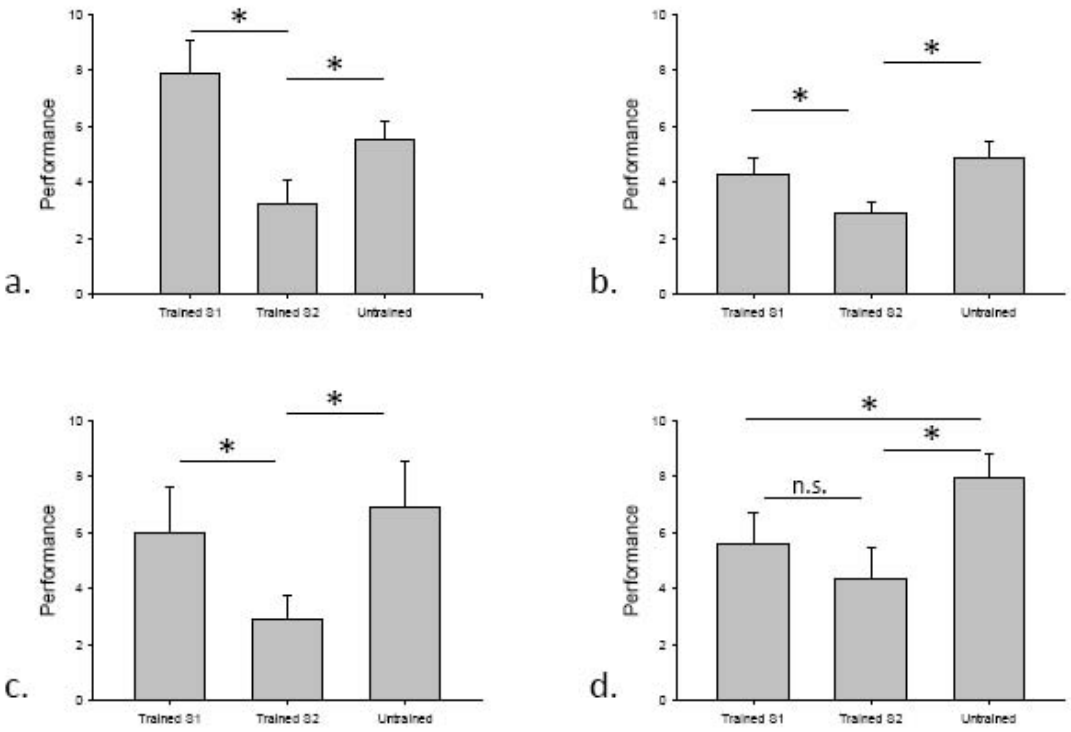

Figure 4.7: Performances of *Rhodnius prolixus* larvae, representing the mean number of trials that were necessary to observe a complete disappearance of the response. Each bar represents either a trained group during its first (S1) or second session (S2) or the associated control group (Untrained) when indicated. (a) 1h retention; (b) 24h retention; (c) 72h retention; (c) 96h retention. Asterisks indicate significant differences ($p<0.05$).

sufficient to evoke the PER (Flores and Lazzari, 1996). Besides, in order to obtain their reward, as mosquitoes and other blood-sucking insects, they need to pierce the skin of their hosts for recovering their food from inside blood-vessels or through the membrane of an artificial feeder. Bugs do not drink blood from drops, neither respond to blood odour nor to cold objects. Furthermore, to perform olfactory conditioning of the PER would have implied to pair an odour delivery (CS) with a thermal stimulation (US) and reinforce the association with a food reward. This means to be able to deliver a warm small amount of blood in a membrane enclosed artificial feeder and to limit the number of trials in order to keep high insects motivation. Despite these particularities, we have recently shown that, if adequately stimulated it is possible to induce olfactory conditioning in *R. prolixus*, both appetitive and aversive, using a classical conditioning procedure (Vinauger *et al.*, 2011a, b).

In the first part of this work, bugs response to an appetitive thermal stimulus displayed a progressive decrease along the repeated presentation of the stimulus. This decrease ended with the complete disappearance of the response. The reappearance of responses after shifting stimulation and ITI temperatures, but keeping the same difference, revealed the central basis of this phenomenon. In other words, disappearance of PER was due to real habituation and not to peripheral processes, such as motor fatigue or sensory adaptation. Furthermore, it is worth mentioning that to evince dishabituation, both the stimulus and the response were identical, except the fact that the intensity of the stimulus was reduced. So, sensory adaptation and motor fatigue could be tested in only one step. This kind of simultaneous control is not always possible in other models, where two steps are necessary. For instance, to test motor fatigue the same response is tested by using a different stimulus (e.g. in olfactory conditioning a different odour) and to test for sensory adaptation the ability of the same stimulus to evoke a different response is measured.

If we centre now our analysis on the insect model itself, our results suggest that the extension of the proboscis, which is easily triggered by heat stimulation (US), is not a fully stereotyped response but a plastic one submitted to the control of superior centres instead.

From an ecological point of view, such a behavioural plasticity appears as highly adaptive, if we take into account the diversity of hot non-host objects that these insects may encounter in their habitat. Some species, like *R. prolixus*, have established a strong association with the human habitat, where warm objects other than hosts are present. These bugs exhibit a high sensitivity to heat that they use to find a potential food source (i.e. endothermic vertebrates) and that is necessary and sufficient to trigger the PER (Flores and Lazzari, 1996). Thus, to be able to stop responding to thermal stimulation that do not provide food seems as adaptive as the possibility to dishabituate the PER to start responding again to other warm objects, because it is essential for their survival.

However, to learn to stop responding to a warm non-edible object is one thing, but to keep reacting in the same way in the future is a different one. Our results show that at least one hour after the habituation procedure, training has an effect on the performances observed during the subsequent habituation procedure. Thus, the habituation procedure induced the formation of a mnesic trace. Twenty four hours later, this effect could not be discriminated from the context effect (data not shown).

Another relevant point from an ecological perspective is the role played by the spatial context in learning. Our results show that the context would be important in the habituation of the PER. In their natural habitat, bugs may encounter many warm objects, some being hosts and the others not. To take into account of the context in which bites are ineffective reduces the possibility of not responding later, when a true host appears in a different place. This link between the learning context and memory have already been demonstrated in other organisms such as the nematode *C. elegans* (Rankin, 2000), the crab *C. granulates* (Hermitte *et al.*, 1999) and in *Aplysia* (Colwill *et al.*, 1988).

The ability of *R. prolixus* to perform other forms of learning has already been demonstrated. Recently, we succeeded in applying Pavlovian conditioning procedures and made bugs associate an olfactory stimulus with either a positive (i.e. appetitive conditioning) or a negative reinforcement (i.e. aversive conditioning), and this applying the association

learned in one context (contingency of an odour with food or punishment) to a different context (spatial orientation) (Vinauger *et al.*, 2011a,b). It must be said, however, that olfactory conditioning of haematophagous is time consuming and submitted to the constraints evoked earlier (e.g. precise control of rewards to keep motivation constant and homogenous across individuals and many others, Vinauger *et al.* 2011a, b). The experimental paradigms based on the PER reveal easier to set up and more adequate to tackle questions that require precise parameter control, in particular, the aversive conditioning of the PER. For this, we used a negative reinforcement (50°C) paired with the PER of bugs responding to an appetitive stimulus (Peltier at 35°C). We observed a more rapid decrease of responses than by simple habituation. Not only insects were able to learn to stop responding in order to avoid the punishment, but they were also able to retain the learned information for 72h. When tested 96h after the first session, the effect of training started vanishing. So, the physiological bases of learning and memory can be studied using a simple and reproducible protocol and along a relatively long time period.

Even if marginally significant ($p = 0.076$), a presumable effect of the context was also observed in this kind of conditioning one hour after training. It consisted in a reduction of the number of trials necessary to stop responding for insects exposed to the context, but not stimulated in the first session (i.e. untrained control group), as compared to the first session of the trained group. This effect was not visible anymore when retention tests were performed 24 or 72h post-training. So, we hypothesize that context memory lasts for less than 24h in *R. prolixus*. Further work would be necessary to confirm or discard this presumption.

We believe that our characterization of the PER of *R. prolixus* is relevant for several reasons. First, because it allows applying a simple, easily reproducible and largely validated learning paradigm to haematophagous insects, vectors of diseases. As indicated before, learning abilities are supposed to play a key role in parasite transmission, but the experimental evidence is scarce because of experimental constrains. Second, once charac-

terized, the PER paradigm furnishes the possibility to explore the neurobiological basis of learning and memory in experimental models biologically and phylogenetically distant from classical ones (i.e. drosophilas and bees). Third, it allows using the same sensory modality, i.e. the thermal one, as US and CS. Four, given the difficulties for setting up simple and reproducible paradigms for studying learning and memory in mosquitoes (by far the most important disease vector insect), the PER of *R. prolixus* offers a model system to analyze biologically relevant questions that concern hematophagy in general. In this sense, it should not be forgotten that even having evolved many times among insects, the haematophagous way of life impose similar selection pressures to all blood-sucking arthropods (e.g. host detection and selection, avoiding of the most defensive ones, etc.).

In this work, we developed an experimental paradigm allowing the in depth study of learning and memory of a disease vector insect. This represents a powerful tool in order to better understand the physiological, molecular and neurobiological mechanisms underlying these cognitive processes, using methodologies developed and widely validated in classical models. We demonstrated that the PER of *R. prolixus* could be successfully habituated and dishabituated, and that it was possible to modify insects' innate response to heat through a conditioning procedure. This new experimental set up thus represents an appropriate controlled context in which questions regarding, for example, molecular basis and circadian modulation of learning and memory can be explored.

4.5 Acknowledgements

The authors are indebted to the staff members of the Neuroethology group for fruitful discussions, to C. Lahondère for critical reading of drafts and to C. Labrousse for technical support with insect rearing. This work received support from the CNRS, the University of Tours and the Agence Nationale de la Recherche (ANR-08-MIE-007, EcoEpi), France. This study was performed as part of the PhD work of C.V. at the University of Tours, under the supervision of C.R.L. and with the support of the French Ministère de la Recherche.

Troisième partie

Chronobiologie de l'apprentissage

En utilisant le paradigme expérimental développé au chapitre précédent, nous nous sommes intéressés aux facteurs endogènes susceptibles d'influencer l'apprentissage et la mémoire des insectes hématophages. En particulier, nous avons testé l'influence du moment de la journée sur les capacités cognitives des triatomines.

Chez ces insectes, de nombreux comportements et processus physiologiques sont sous l'influence d'horloges circadiennes (Barrozo *et al.*, 2004). Notamment l'activité est modulée au long de la journée. Les insectes recherchent activement leurs hôtes lorsque ces derniers sont inactifs, optimisant ainsi leur fitness. Comme discuté dans les chapitres précédents, être capables d'apprendre et mémoriser des informations au sujet des hôtes est hautement adaptatif, mais l'être dans le contexte temporel approprié l'est d'autant plus. En effet, conserver une trace mnésique d'informations apprises dans la journée, représente une dépense inutile d'énergie.

En élevant des individus sous un cycle 12:12 L:D et en réalisant les sessions d'entrainement et de test à différents moments du jour subjectif, nous avons pu montrer que la capacité de *Rhodnius prolixus* à mémoriser l'information, et non la rétention ou l'utilisation de l'information mémorisée, est modulée par le système circadien. Les performances étant maximales en début de nuit, lorsque l'insecte est à la recherche d'un hôte sur lequel s'alimenter. En répétant ces expériences avec des insectes maintenus en obscurité constante, nous avons pu mettre en évidence l'implication d'horloges endogènes et donc démontrer la nature véritablement circadienne du rythme observé. Ces résultats confirment l'importance de la prise en compte du moment du jour subjectif dans l'étude des capacités d'apprentissage des insectes hématophages et constituent seulement la deuxième démonstration d'une telle modulation chez les insectes en général (Decker *et al.*, 2007). De plus, ils révèlent la nécessité de poursuivre les investigations concernant les divers facteurs susceptibles d'interagir avec les capacités cognitives des insectes vecteurs de maladies.

Article en préparation: Vinauger, C. & Lazzari, C. R. (*in prep.*)

Chapitre 5

Circadian modulation of learning abilities in a disease vector insect, *Rhodnius prolixus*

Abstract. It has been largely admitted that the cognitive abilities of disease vector insects may have drastic consequences on parasites transmission. However, despite the research effort that has been invested in the study of learning and memory in haematophagous insects, hitherto, few conclusive results were obtained. The application of the classical paradigm of the conditioning of the proboscis extension response (PER) has been recently shown to constitute an appropriate bioassay to study the cognitive abilities of the haematophagous bug *Rhodnius prolixus*. Using the aversive conditioning of the PER as learning protocol, we tested for the first time in a haematophagous insect, whether or not the ability of bugs to learn and remember information varies along the day and the origin of such variation. Here we show that: 1) the ability of bugs to learn and remember information is submitted to a diel rhythm; 2) the performance is maximal at the beginning of the night, i.e. when insects seek for a host to feed on; 3) the learning daily rhythm is a true circadian one, i.e. it is ruled by an endogenous clock and 4) the process submitted to the control of the circadian system is the capability to acquire information and not that to retrieve it. These results are the first ones to unravel the importance of the circadian system on the learning abilities of blood-sucking insects. Furthermore, they confirm that PER conditioning is a powerful tool that makes possible the analysis of the cognitive abilities of haematophagous bugs, opening novel research avenues to be explored.

Key words: Learning, memory, PER, circadian clocks, triatomines, Chagas disease.

5.1 Introduction

Numerous human and animal diseases are transmitted by insects which vectorial capacity, i.e. their ability to transmit the disease, can be modulated by various factors. Some of them have been well characterised (for instance, vectors density or host defensive behaviour; Kelly and Thompson, 2000) and it has been admitted that the cognitive abilities, and more precisely the ability to learn and memorise information, should have a great epidemiological impact. However, until recently, such abilities were not clearly experimentally evinced, insomuch that some authors even wondered if haematophagous insects would be able to learn anything from their hosts (Alonso *et al.*, 2003). In recent years, the number of studies devoted to unravel the learning abilities of blood-sucking insects has increased concerning mosquitoes (McCall and Eaton, 2001; McCall *et al.*, 2001; McCall and Kelly, 2002; Kaur *et al.*, 2003; Alonso and Schuck-Paim, 2006; Tomberlin *et al.*, 2006), tsetse flies (Bouyer *et al.*, 2007) and triatomine bugs (Vinauger *et al.*, 2011a, b). These studies have provided direct or indirect evidences of learning abilities but, to our knowledge, none of them dove deep enough on the characterization of learning and memory, for instance, on the factors that could modulate those abilities.

In a large number of vector species, activity is modulated along the day and patterns have been well described. By actively seeking for hosts to feed on, when the latter are inactive, these insects have found a way to optimize their fitness (directly related to their feeding success). To learn and remember information about hosts is highly adaptive, but to be selective in learning only in the proper temporal contexts even more, because to remember occasional events occurring at daytimes that are not relevant, represents a waste of energy and information processing. Thus, it would be adaptive for them to be able to learn and remember information about their hosts only in the temporal context corresponding to host seeking and not in non relevant temporal contexts. In addition, since learning and memory are two processes that imply a significant cost for insects, in terms of energy, we would expect that these abilities would be used more efficiently in the period of time

when they are salutary for them. Up to date, in insects the only known demonstration of circadian regulation of learning abilities concerns olfactory conditioning in the cockroach *Leucophaea maderae* (Decker *et al.*, 2007).

To analyze whether or not the case of *Leucophaea maderae* can also be generalized to haematophagous insects, we chose as experimental model *Rhodnius prolixus*. These hemimetabolous and obligatory blood-feeders are easy to rear in the laboratory and their motivational state can be well controlled (Bodin *et al.*, 2009a, b). Furthermore, it is a classical model in insect physiology since the seminal work of V.B. Wigglesworth in the 1930's. Another specificity of these insects is their stereotyped response to proximal thermal stimulation, i.e. the proboscis extension response (PER). This represents an advantage in comparison to mosquitoes or biting flies, since it makes possible to adapt experimental tools and paradigms developed in classical models for the study of learning in insects as honeybees or fruit-flies, which is PER conditioning (Chabaud *et al.*, 2006; Giurfa, 2007; Carcaud *et al.*, 2009; Strube-Bloss *et al.*, 2011). By rearing bugs under a 12:12 Light:Dark regime and by training and testing the insects at different time of the subjective day, we tested whether or not the ability of *Rhodnius prolixus* to learn and recall information is modulated along the day, as well as the endogenous or exogenous origin of this modulation.

5.2 Material and Methods

5.2.1 Insects

Fifth-instar larvae of *Rhodnius prolixus* were used throughout the experiments. Bugs were reared in the laboratory under a 12h:12h light:dark illumination regime, at 25 ± 2°C and 50-70 % relative humidity (RH). Lights switched on at CT0 and switched off at CT12. Insects were fed weekly on sheep heparinised blood, using an artificial feeder (Núñez and Lazzari, 1990). Fifth-instar larvae that had just moulted were isolated in individual plastic containers and starved until being tested, 15 days after their moult.

5.2.2 Experimental apparatus

Insects were tethered by their dorsal thorax to a stiff steel wire, using double-sided adhesive tape, in an experimental room which temperature was kept at 25 ± 2°C. A Styrofoam ball was placed between their legs in order to provide them tarsal contact and, this way, to reduce unnecessary stress. A Peltier element, representing an accurate and controllable heat source, was placed in front of animals, at a distance from which they could contact the surface while extending their proboscis. This element allowed rapid temperature changes of the surface that was presented to the insects and, this way, we could display an appetitive heat source (35°C) or apply a negative reinforcement (50°C) (Fig. 5.1).

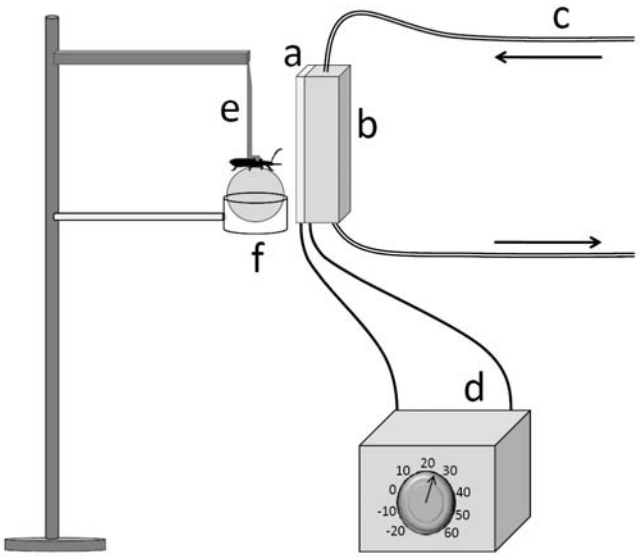

Figure 5.1: Experimental device used for training the PER of *R. prolixus*. It allows the delivery of thermal stimulation. The Peltier element was used to deliver the appetitive and the aversive stimulation. a, Peltier element; b, Aluminium heat dissipator; c, Enclosed water based cooling; d, Thermostat; e, steel wire allowing the fication of an insect; f, Styrofoam sphere (1 cm diameter).

The efficiency of the Peltier element was improved by a water-cooling system placed on the back of the apparatus to cool down the dissipated heat. This way, the element temperature could switch from 25°C to 35°C and from 35°C to 50°C in less than a second. A thermal sensor was placed in contact with the Peltier element and used to control the

temperature of the device.

The assays were monitored with the aid of an infrared-sensitive camera provided with an array of infrared LEDs (emission 900 nm). This light illuminated the scene without being perceived by the bugs (Reisenman *et al.*, 1998) and allowed us to observe proboscis movements with more details.

5.2.3 PER conditioning paradigm

We carried out aversive conditioning experiments, testing the ability of *R. prolixus* to inhibit its proboscis extension response (PER) when trained to receive a thermal shock (50°C) if responding to a thermal stimulus (35°C) and extending its proboscis.

The temperatures used in these experiments correspond to thermal preferences and tolerance of these insects (Okasha, 1968a, b; Schilman and Lazzari, 2004). The appetitive stimulus temperature was fixed at 35°C because it corresponds to hosts' skin surface temperature and it has been shown that it elicits the PER. Concerning the negative reinforcement, 50°C represents an aversive but not lethal temperature, and is ecologically relevant since such temperatures can be encountered by the insects in their natural environment (temperatures above 60°C can be observed inside and outside houses, as. cooking elements or stones exposed to sunlight, etc.).

At the beginning of each experiment, insects were placed individually in the experimental apparatus for a 30s period, in order to let them familiarize with the experimental situation. For each training or testing situations, animals that did not respond by extending their proboscis during the two first stimulations were considered as not motivated to feed and thus discarded from the analyses. A PER was recorded when the proboscis was fully extended, i.e. when it was positioned horizontally, making a 180 ° rotation from its original position.

During the tests, we observed the occurrence or absence of the PER at 35°C, and we calculated the percentage of insects responding to heat stimulation. Each individual was

repeatedly confronted to stimulation trials until complete disappearance of the response, i.e. three successive trials without any answer.

5.2.4 Experimental procedure

Each experimental group was confronted to two experimental sessions, a training one and a test one. Different groups were constituted, experimental (trained) and control ones as detailed below. Insects of the experimental groups underwent two sessions, a training one and a test one (Fig. 5.2). During each session, bugs were submitted to repeated trials, after a 30s familiarization period. One trial being defined as follow:

(a) Appetitive stimulation (35°C) during 10s.

(b) In case of PER, i.e. insects responding to the thermal stimulation, a heat shock was delivered to the extended proboscis at the end of the 10s period, by increasing the temperature of the Peltier to 50°C. If no PER was displayed, at the end of the 10s stimulation, insects did not received any reinforcement.

(c) Inter-trial interval of 50s.

In other words, during the different sessions, each individual was placed in front of the Peltier element which temperature was fixed at 25°C, corresponding to the ambient temperature of the experimental room. The insects were left without stimulation for 30s in order to familiarize with the experimental situation. Then, they were submitted to several successive trials, separated by 50s inter trial intervals. During these intervals, the Peltier's temperature was maintained at 25°C.

5.2.5 Conditioning experiments

a. Effect of subjective time on the ability to acquire and recall memory.

To determine whether the circadian phase regulates the ability of these animals to form and recall memory, we trained and tested insects at two different moments of the subjective

day. A first group (group 1; n= 14) was trained and tested 24h later at Circadian Time (CT) 14 (i.e. early scotophase, early subjective night) a second one (group 2; n= 17) was submitted to the same procedure at CT2 (i.e. early photophase, early subjective day).

b. Effect of subjective time on the ability to acquire and/or to recall memory.

The ability to perform in the test situation depends on the ability of the insects to acquire and consolidate a memory during the training session and to recall the memory during the testing session. To test if one or both processes could be modulated, we trained insects during the first part of the subjective night and tested them 12h later, during the first part of the subjective day (group 3 CT14-CT2; n= 31). In the same way, we trained insects during the first part of the day and tested them 12h later, during the first hours of the night (group 4 CT2-CT14; n= 15).

c. Daily modulation of behavioural response to heat stimulation.

To discard any interference of temporal modulation of response to heat, we also quantified the proportion of insects responding to appetitive stimulation (35°C) at two different circadian times: CT2 (n= 54), i.e. beginning of subjective day, and CT14 (n= 71), i.e. beginning of subjective night.

d. Assessing the implication of the circadian system.

Insects were maintained during 3 days under 12:12h L:D regime and then placed under constant darkness (D:D) for another three days. Training occurred on the second day of constant darkness and testing on third day of constant darkness. Experiments were conducted under functional darkness in order to avoid as possible to give any *Zeitgeber* to bugs.

A first group was trained and tested at the beginning of the subjective night (CT14; n= 15) and another one at the beginning of the subjective day (CT2; n= 15). The time

of test periods were adjusted to 23h post-training in order to compensate for the expected shortening of the free running period under D:D conditions (Lazzari, 1992) and to confirm that the absence of a response under D:D was not due to a more important shift of the rhythm under free-running conditions.

For each experiment, the performance of the insects was also compared to a control group, whose individuals were not trained during the first session, i.e. they were placed in the set up and exposed to a constant temperature of 25°C, during the mean time of a training session (determined as the mean time necessary to observe complete disappearance of the PER in the respective trained groups). Insects of the control groups were then tested in a second session, as the associated trained groups.

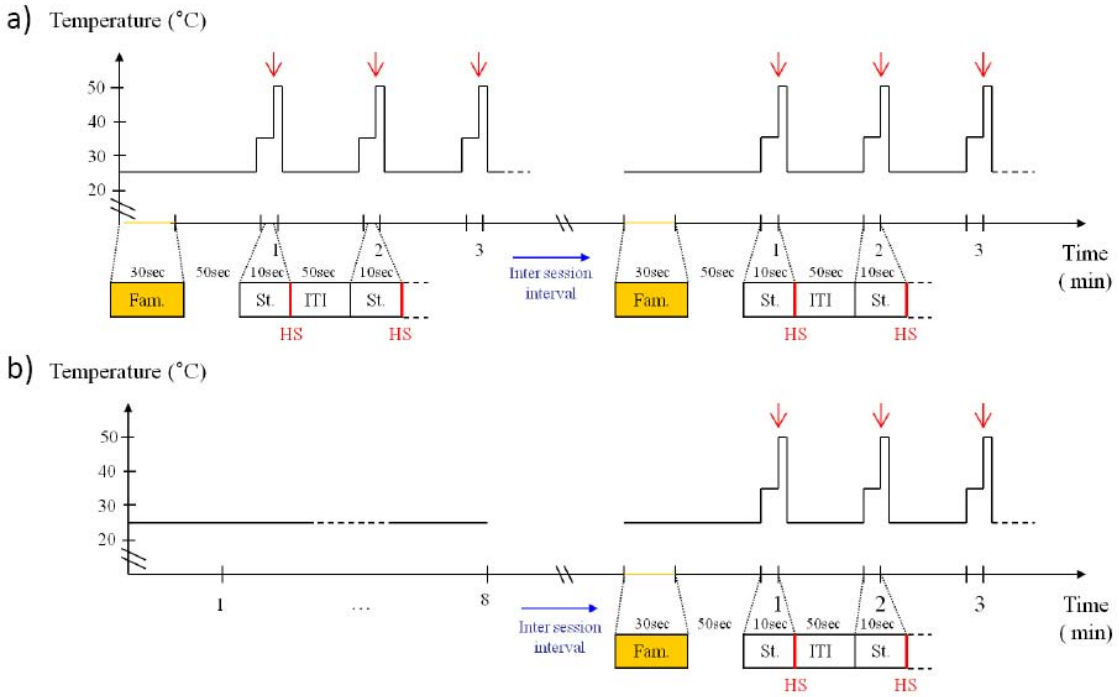

Figure 5.2: Sequence of event delivery (i.e. appetitive thermal stimulation and inter-trial interval) during training sessions of the different experimental groups: (a) Procedure employed fo trained groups; (b) Procedure employed for control (untrained) groups.

5.2.6 Data analysis

Learning performance of individual insects was quantified by determining the number of trials required to observe the disappearance of the response in three successive trials (Braun and Bicker, 1992). A mean performance was then calculated for each group. Wilcoxon tests for paired data was used to compare performances between training (**S1**) and test (**S2**) sessions and comparison between the performances of test and control groups were made by using Mann-Whitney test for independent data. All statistical tests were computed using R software (R Development Core Team, 2010) and significances were considered at $\alpha = 0.05$.

5.3 Results

a. Effect of subjective time on the ability to acquire and recall memory. At CT14 insects were able to use the learned information to stop responding more rapidly during the test session (S1: 4.2 ± 0.5 trials; S2: 2.9 ± 0.3 trials; Wilcoxon test: $p = 0.0053$), whereas at CT12 no influence of training was observed during the test session (S1: 7.5 ± 1.2 trials; S2: 7 ± 1.1 trials; Wilcoxon test: $p = 0.24$), revealing a daily modulation of learning and memory. The influence of the context on results observed at CT14 can be discarded since no there was no significant difference between performance of naïve bugs (trained, S1) and control insects (control: 4.8 ± 0.6 trials; Mann-Whitney test: $p = 0.42$). In addition, performance of trained bugs was significantly better (i.e. less trials were required to observe the disappearance of PER) than performance of control insects (Mann-Whitney test: $p = 0.011$). Results are depicted in Fig. 5.3a and b.

b. Effect of subjective time on the ability to acquire and/or to recall memory. In order to discriminate whether the observed modulation is due to a modulation of the ability to acquire the information or to a modulation of the ability to recall the learned information, two groups were tested (Fig. 5.3c and d).

Insects of the first one, trained at CT14 and tested at CT2, were able to recall the learned information and required less trials to stop responding during the second session (S1: 8.7 ± 0.6 trials; S2: 3.2 ± 0.6 trials; Wilcoxon test: $p<0.0001$). They also required fewer trials than control insects (control: 7.7 ± 0.6 trials; Mann-Whitney test: $p<0.0001$). No difference was observed between naïve insects (trained group, S1) and control bugs (Mann-Whitney test: $p= 0.33$).

On the other hand, when trained at CT2 no effect of training was observed on the performance of bugs tested at CT14 (S1: 6.5 ± 0.9 trials; S2: 7.2 ± 1.2 trials; Wilcoxon test: $p= 0.68$), revealing that the modulation occurs at the acquisition level and not for the recall of learned information.

c. Daily modulation of behavioural response to heat stimulation. However, the results obtained for bugs trained at CT14 and tested and CT2 could also be explain by a daily modulation of *Rhodnius* motivation to respond to heat. The reduced number of trials observed at CT2 would then be due to a lack of motivation to respond and not to individual experience. To test this hypothesis, we tested the response of naïve bugs to appetitive stimulation at two circadian times: CT2, i.e. early photophase, and CT14, i.e. early scotophase. We observed that insects responded significantly more during the early scotophase (83.1 % ($n= 71$) against 44.4 % at CT2 ($n= 54$); Fisher's Exact Test: $p<0.0001$; Fig. 5.4a).

Thus, in order to discriminate the effect heat response modulation from actual learning in bugs trained at CT14 and tested at CT2, we discarded insects that did not respond during the second session, keeping only those which were motivated to respond to heat. After this correction of data, the difference between S1 and S2 remained significant (S1: 9.9 ± 1.1 trials; S2: 5.6 ± 1.1 trials; Wilcoxon test: $p= 0.0015$; Fig. 5.4b), revealing an actual effect of training.

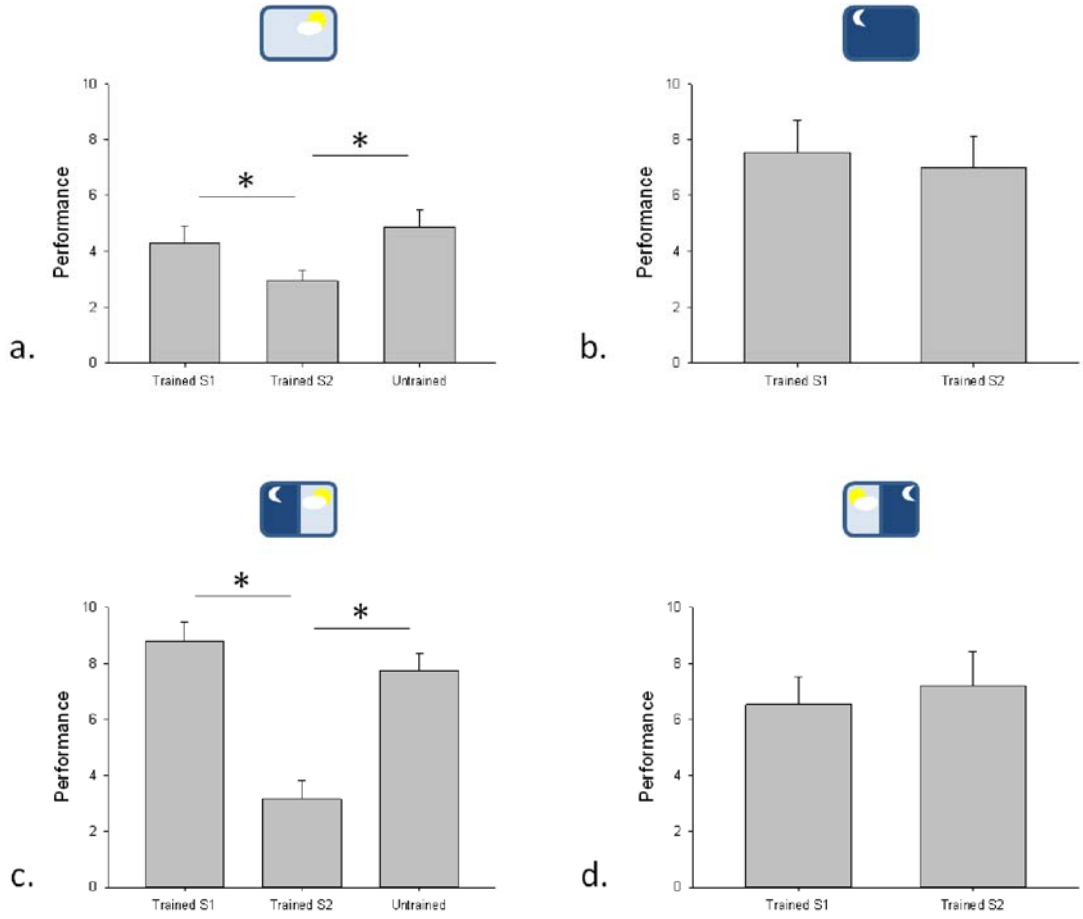

Figure 5.3: Performances of *Rhodnius prolixus* larvae, (defined as the mean number of trials that were necessary to observe a complete disappearance of the response). Each bar represents either a trained group during its first (S1) or second session (S2) or the associated control group (Untrained) when indicated. (a) Performances of insects trained and tested at CT14; (b) Performances of insects trained and tested at CT2; (c) Performances of insects trained at CT14 and tested at CT2; (d) Performances of insects trained at CT2 and tested at CT14. Asterisks indicate significant differences ($p<0.05$).

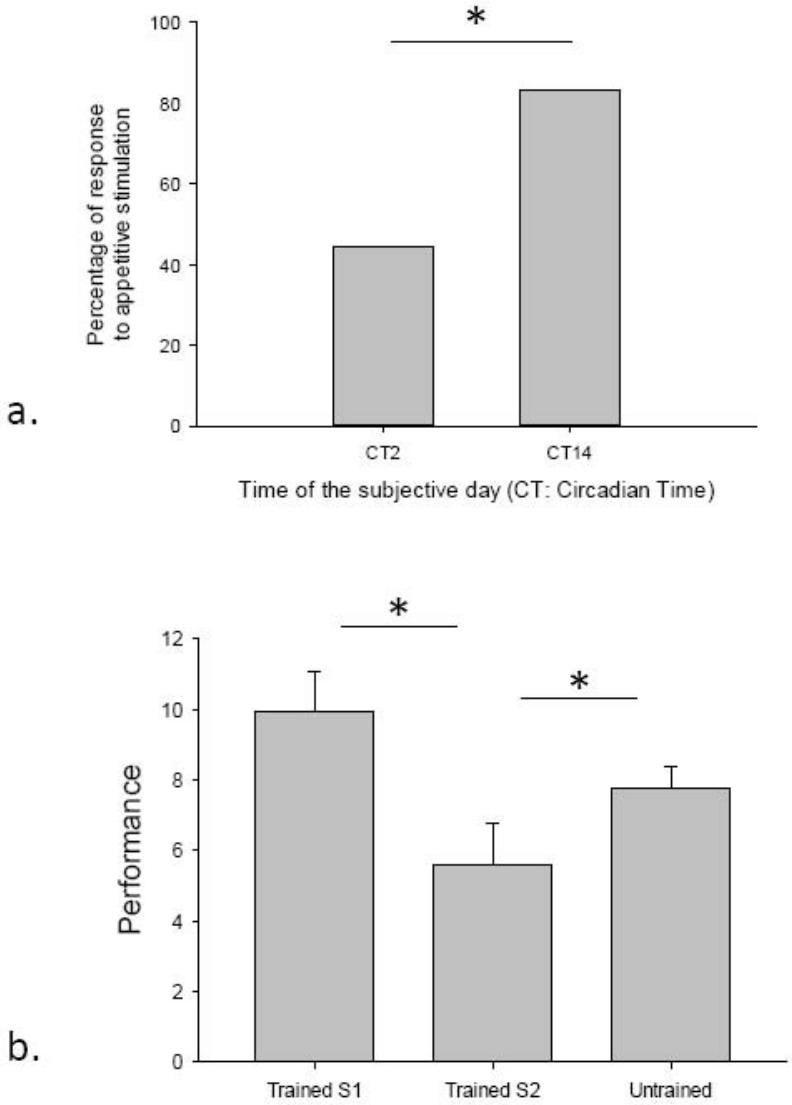

Figure 5.4: a. Percentage of insects responding to thermal appetitive stimulation at two different moment of the day: the beginning of the day (left bar) and the beginning of the night (right bar). b. Performances of *Rhodnius prolixus* larvae (defined as the mean number of trials that were necessary to observe a complete disappearance of the response), that were trained at CT14 and tested at CT2. Each bar represents either a trained group during its first (S1) or second session (S2) or the associated control group (Untrained). Asterisks indicate significant differences.

d. Assessing the implication of the circadian system. To assess the implication of the circadian system in the modulation of learning abilities, two further groups were tested while kept under constant darkness. The first one was trained at CT14 and tested 23h later (Fig. 5.5a). Bugs required significantly less trials during the second session than during the first one (S1: 5.7 ± 0.6 trials; S2: 3.2 ± 0.5 trials; Wilcoxon test: p= 0.0002) and less trials than untrained insects (control: 5.1 ± 0.3 trial; Mann-Whitney test; p= 0.0021), revealing a clear effect of training. No difference was observed between performances of untrained and naïve bugs (Mann-Whitney test: p= 0.86).

The second group was trained at CT2 and tested 23h later (Fig. 5.5b). This time no effect of training was evinced (S1: 4.8 ± 0.4 trials; S2: 5.2 ± 0.6 trials; Wilcoxon test: p= 0.76), revealing that the modulation was maintained even in the absence of *Zeitgeber*.

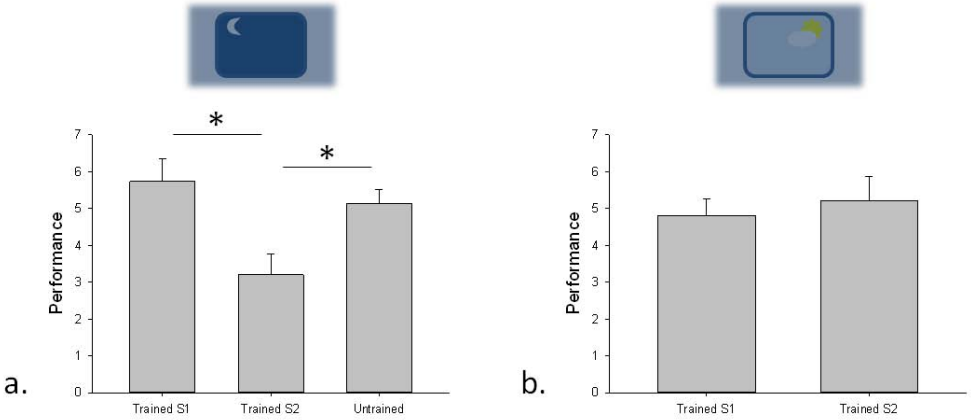

Figure 5.5: Performances of *Rhodnius prolixus* larvae (defined as the mean number of trials that were necessary to observe a complete disappearance of the response) maintained under constant darkness. Each bar represents either a trained group during its first (S1) or second session (S2) or the associated control group (Untrained) when indicated. (a) Performances of insects trained and tested during the subjective early scotophase (CT14); (b) Performances of insects trained and tested during the subjective early photophase (CT2). Asterisks indicate significant differences (p<0.05).

5.4 Discussion

Our results demonstrate that learning and memory in the triatomine bug, *R. prolixus*, are processes modulated by the circadian system, and that the effectiveness of conditioning is strongly dependent on the circadian phase. Indeed, when trained and tested during the subjective scotophase bugs were able to use their individual experience (training) in order to stop responding more rapidly during the second session and thus to avoid punishment. Conversely, when trained and tested during the subjective photophase, no effect of training was observed. However, as said above, the process of learning and memory is composed of several distinct steps (i.e. acquisition, consolidation, retention, retrieval and performance; Margulies *et al.*, 2005) on which the circadian phase could have an impact. In the experimental paradigm that was developed here, we were able to discriminate at least between two hypotheses. The different performances observed at night and during the day were due to either a deficit in bugs' ability to acquire the memory or to an impossibility to recall and use the memories in order to adjust their behaviour. Results obtained with bugs trained at CT14 and tested at CT2 discarded the first hypothesis since *R. prolixus* performed equally well in the early photophase and early scotophase when trained at CT14. Congruently, when trained at CT2, i.e. the early photophase, bugs failed to perform in both the early photophase and the early scotophase. Put in other words, these results revealed that 1) the ability to recall learned information was independent of the circadian time and 2) that training time was not a necessary contextual cue, required for performance (time stamping). It worth mentioning that similar phenomenon was observed in the cockroach *Leucophaea maderae* (Decker *et al.*, 2007).

Insects trained at CT2 were either unable to form new memories (acquisition) or to consolidate and retain the learned information for 12h. A further experiment, where testing would occur less than 1h post training, might shed some additional light on this question. In vertebrates (mice), the early long term potentiation in the hippocampus is under the control of the circadian system (Chaudhury *et al.*, 2005) but in *Aplysia*, the circadian sys-

tem has been shown to modulate long term memory only. The formation of short term memory seems to be independent of the time of the day (Fernandez *et al.*, 2003; Lyons *et al.*, 2006). In insects, Decker *et al.* (2007) demonstrated that cockroaches trained at CT2 failed to perform even 5-30 min post-training, suggesting that the modulation by the circadian system took place during the earliest stages of memory formation.

In the present study daily modulation might have occurred at another level, directly on the response to thermal stimulation. Indeed, results of experiment *c.* showed a clear fall in the percentage of insects extending their proboscis during the early photophase (44.4 % against 83.1 % during the early scotophase). When both training and testing occurred at CT2, this modulation was not biasing the data since only insects that were motivated or able to respond to heat were selected. However, in the group of insects trained at CT14 and tested at CT2, the selection was made at CT14, where non-responding bugs were discarded. Thus, while testing at CT2, some insects might have displayed fewer responses not because of training, but simply because they were less sensitive or less motivated to respond than at CT14. To discriminate between these explanations, we removed from analysis insects that were not responding during the second session (i.e. at CT2). This correction of data revealed an actual effect of training. Thus the higher difference that was observed before correction was due to the cumulative effects of learning and to the modulation of the response to heat.

To assess the involvement of an endogenous clock, we repeated the first experiment (exp. a.) but this time, bugs were kept under constant darkness conditions to let the circadian system free run. Our results indicate that the rhythm in learning and memory performances is under the control of an endogenous oscillator. The persistence of the rhythm under constant darkness demonstrates that it is self-sustained and therefore truly circadian. This is, to our knowledge only the second demonstration of such circadian modulation of cognitive abilities in insects (see Decker *et al.*, 2007). The fact that the

adjustment of the free running period (23h instead of 24h) led to conclusive results, suggests that the period of the rhythm under D:D is shorter than the period of the rhythm under L:D conditions. This was already observed for other circadian rhythms already described in this species and was expected for a nocturnal animal (Aschoff, 1989).

in addition, we also analyzed bugs responses to heat under constant darkness. In these conditions 65 % of bugs extended their proboscis when stimulated at 35°C during the subjective photophase (n= 23), whereas 86 % responded during the early photophase (n= 35). This difference was less pronounced than under 12:12 L:D illumination regime and rendered only marginally significant results (Fisher's Exact Test: p= 0.06). However, in both cases those data were obtained after the analysis of experimental groups. To conclude on the implication of endogenous clocks in the modulation of response to heat thus requires further specific experiments.

Regarding the adaptive value of such temporal modulation of learning abilities, some remarks need to be made. The results obtained in the present study corroborate most examples found in the literature, where circadian modulation of memory formation has been shown. The optimal performances are observed during the active phase of the tested animal (Lyons *et al.*, 2006). That would mean that, as formulated by Decker *et al.* (2007), memories are only beneficial when formed in the environmental (including temporal) context in which they will be used. In the case of *R. prolixus*, which are nocturnal insects, environment and activities are highly periodic (Ampleford and Steel, 1982; Constantinou, 1984; Lazzari, 1992; Reisenman *et al.*, 1998, 2002; Barrozo *et al.*, 2004; Bodin *et al.*, 2008), most of them being controlled by circadian clocks. Thus, because this haematophagous species is almost exclusively active at night and spends the day time at rest inside shelters, it would not be profitable to form memories of the daytime environment. These latter would influence bugs' activities based on information obtain at a time and in an environment in which none of nocturnal activities are performed. As suggested by Decker *et al.* (2007) those memories would even interfere with successful foraging during night

conditions. Furthermore, the molecular and neurobiological processes that are involved in memory acquisition are not costless. The formation of a new memory implies expending a non negligible amount of energy and a cost in terms of neural tissue and connections devoted to the storage of the memory. Thus, to avoid forming new memories in time where they would not be relevant is also adaptive in terms of energy.

In the context of disease vectors study, our results highlight the importance of the temporal context when dealing with learning and memory. Past failure in this field of investigation might have suffered from a lack of control regarding the time of day during which experiments were conducted. In summary, these results add another element to explain the high evolutionary success of triatomine bugs and their efficiency as disease vectors. These results also bring more knowledge, making possible to dive deeper in the understanding of learning and memory in haematophagous insects.

From a more general point of view, this represents the second demonstration of circadian regulation of insect learning. In their foray, Decker *et al.* (2007) evinced a modulation of cockroaches' olfactory learning, using a differential conditioning procedure. Here we demonstrated that the thermal learning of phylogenetically distant insect is also regulated by the circadian system, applying methodologies adapted from operant conditioning procedures. We are thus in presence of what seems to be a wide spread phenomenon, involving different insect models and different types of learning. Taken together, both studies (Decker *et al.*, 2007 and this paper) reveal the importance of taking into account the subjective moment of the day when dealing with insect learning abilities.

5.5 Acknowledgements

The authors are indebted to the staff members of the Neuroethology group for fruitful discussions, to C. Lahondère for critical reading of drafts and to C. Labrousse for technical support with insect rearing. This work received support from the CNRS, the University of Tours and the Agence Nationale de la Recherche (ANR-08-MIE-007, EcoEpi), France. This

study was performed as part of the PhD work of C.V. at the University of Tours, under the supervision of C.R.L. and with the support of the French Ministère de la Recherche.

Conclusion

L'apprentissage et la mémoire, définis comme la modification du comportement d'un animal basée sur des expériences passées et le stockage des informations expérientielles (Lorenz, 1981), permettent aux animaux de faire face à des environnements variables et d'optimiser leur fitness. Ces processus cognitifs ont été décelés chez de nombreux organismes, y compris chez les invertébrés. Certaines espèces d'insectes, l'abeille et la drosophile, sont même devenues des modèles invertébrés classiques pour l'étude de l'apprentissage. Les travaux réalisés chez ces insectes ont permis de dévoiler les mécanismes génétiques (Brembs, 1996 ; Tully *et al.*, 1994), moléculaires (Sutton *et al.*, 2002), neurobiologiques (Menzel et Giurfa, 2001 ; Hourcade *et al.*, 2009, 2010) et évolutifs (Mery, 2006, 2007 ; Mery *et al.*, 2007), associés aux capacités d'apprentissage.

Jusqu'à récemment, peu d'informations étaient disponibles au sujet des capacités cognitives des insectes hématophages, responsables de la transmission de nombreuses maladies animales (Alonso, *et al.*, 2003). Pourtant, d'un point de vue évolutif, il eût été surprenant que ces insectes au mode d'alimentation si particulier ne soient pas capables d'apprendre et mémoriser des informations leur permettant de faire face à la variabilité de leur environnement (McCall et Kelly, 2002). Les multiples adaptations dont les hématophages font preuve, témoignent de l'intensité des pressions de sélection qui s'exercent au niveau individuel. Ces insectes doivent en effet se procurer un aliment qui circule à l'intérieur de vaisseaux sanguins, dissimulés sous la peau d'animaux vertébrés mobiles qui peuvent jouer à la fois le rôle de proies et de prédateurs. L'adaptation à la vie hématophage a également été possible grâce à diverses adaptations physiologiques. Être capable de digérer du

sang implique de pouvoir gérer les stress oxydatif produit par les radicaux hèmes issus de l'hémoglobine, le stress hydrique lié à l'absorption d'un liquide riche en eau, d'héberger des symbiotes permettant d'acquérir des éléments nutritifs essentiels. Différentes stratégies physiologiques et métaboliques ont permis aux hématophages de surmonter ces difficultés. Du point de vue comportemental les insectes vecteurs ont mis en place des stratégies visant à optimiser les chances de trouver un hôte sur lequel s'alimenter, tout en minimisant les risques d'être blessé ou tué au cours de l'opération (modulation de la réponse aux signaux de l'hôte par le système circadien (Bodin *et al.*, 2008) et l'état physiologique (Klowden, 1990 ; Bodin *et al.*, 2009a, b)). Les études menées sur le terrain ont permis d'envisager qu'en plus des adaptations décrites jusqu'à présent, ces insectes pourraient être capables d'affiner leurs réponses innées aux signaux des hôtes grâce à l'apprentissage et la mémoire.

Cependant, l'apprentissage étant un processus complexe, il est parfois difficile de le dissocier clairement d'autres processus qui peuvent conduire, de manière circonstancielle, à des réponses comportementales similaires. C'est notamment pour cette raison que le cadre expérimental et conceptuel, nécessaire à la démonstration de l'apprentissage, est très exigeant. La nécessité de respecter ces contraintes pour démontrer l'existence de capacités d'apprentissage s'explique notamment par la dimension biologique des processus impliqués. À la différence des phénomènes "immédiats", l'apprentissage induit la formation d'une trace mnésique plus ou moins pérenne, qui confère un avantage adaptatif à l'individu en lui permettant de faire face à son environnement.

Par conséquent, du fait de cet avantage, la question qu'il parait raisonnable de poser est d'avantage celle des conditions expérimentales permettant de révéler et caractériser les capacités d'apprentissage d'un organisme, que celle de savoir si ce dernier en est dépourvu ou non. Il s'agit alors de déterminer jusqu'à quel point les capacités cognitives se sont développées au cours de l'évolution d'un animal donné et d'évaluer leur impact sur la biologie de l'espèce en question et ses interactions avec d'autres organismes. Pour ces raisons, il est crucial de distinguer l'apprentissage de tout autre processus physiologique ou comportemental.

Conditionnement olfactif.

Dans un premier temps nous nous sommes employés à adapter une procédure de conditionnement classique olfactif aux contraintes liées aux hématophages. Cette première étape n'ayant pas été respectée dans les travaux réalisés par Abramson *et al.* (2005) et Aldana *et al.* (2008), ces derniers n'étaient pas parvenus à mettre en évidence les capacités d'apprentissage de la punaise hématophage *R. prolixus*. L'objectif de leurs travaux était de tester la capacité de ces punaises à associer une odeur avec la présentation d'un objet chaud (un alimentateur artificiel contenant une solution saline). Les odeurs utilisées par ces auteurs été choisies car n'ayant aucune signification biologique a priori pour les insectes. En réalité, la perception de ces odeurs par le système sensoriel des punaises n'a pas été préalablement vérifiée. Il se pourrait alors que les résultats négatifs obtenus ne soient pas dus à l'incapacité de ces insectes à réaliser une association entre deux stimuli, mais plutôt au fait que le stimulus conditionnel employé n'ait pas été perçu par les insectes. De plus, en l'absence de phagostimulants dans la solution saline, il semble qu'aucune récompense n'ait été véritablement délivrée.

Afin de surmonter les difficultés rencontrées par ces auteurs, nous avons utilisé l'acide L-lactique en tant que stimulus conditionnel (CS). Cette odeur est dite comportementalement "neutre" puisqu'elle ne déclenche ni attraction, ni répulsion lorsqu'elle est présentée seule. En revanche, il s'agit d'un composé volatile perçu par les insectes (Barrozo et Lazzari, 2004b) et qui n'est pas extérieur au contexte biologique de ces insectes. L'acide lactique est en effet émis par les hôtes vertébrés, au travers de la peau, par transpiration (Acree *et al.*, 1968; Bernier *et al.*, 1999, 2000). L'entrainement des insectes à été réalisé dans un alimentateur artificiel conçu pour cette expérience et permettant d'entrainer simultanément plusieurs individus. Ce dispositif offre la possibilité de délivrer l'odeur de façon contrôlée et d'apparier cette diffusion avec la présentation d'un repas sanguin. A chaque essai, les individus n'ont reçu qu'un repas partiel contrôlé (15 µL) de façon à constituer une véritable récompense sans pour autant induire une inhibition du comportement de réponse aux

signaux de l'hôte (Bodin *et al.*, 2009b), c'est-à-dire à la chaleur et aux odeurs associées à la présence d'un hôte. Les tests comportementaux ont été réalisés dans un olfactomètre. Guidés par une anémotaxie positive, les insectes se sont engagés dans le labyrinthe et, une fois à l'intersection entre les branches, ont eu à choisir entre un courant d'air ambiant et un courant d'air chargé en acide lactique, dans les mêmes concentrations que lors de l'entrainement.

Les résultats obtenus démontrent que la punaise *R. prolixus* est bel et bien capable d'associer un stimulus neutre (l'odeur) avec un renforcement positif (repas sanguin).

L'association de l'acide lactique (CS) avec la chaleur (US) associée au repas sanguin, a conduit à une modification de la réponse comportementale vis-à-vis de l'acide lactique.

Pour les insectes, cette capacité de modifier leur réponse à des signaux neutres en fonction de leur expérience individuelle s'avère très adaptative. Elle signifie être capable d'apprendre et associer une odeur avec la possibilité de trouver un hôte et donc d'obtenir un repas sanguin. Cependant, comme souligné précédemment, les hôtes peuvent également jouer le rôle de prédateurs de part leur comportement défensif et antiparasitaire. Il parait donc tout aussi adaptatif d'être capable d'associer une odeur avec la possibilité d'être blessé ou tué par le comportement d'un hôte qui serait trop défensif.

Afin de tester cette hypothèse, nous avons développé un dispositif permettant la présentation d'un signal olfactif et d'un choc mécanique, tout en garantissant une contingence temporelle entre les deux stimuli. Le choc mécanique a été paramétré de façon à représenter une stimulation aversive (libération de phéromones d'alarme chez des individus adultes) sans blesser les insectes (pas de blessures visibles, pas d'impact sur la survie des individus). Le test comportemental est cette fois-ci réalisé dans un compensateur de locomotion de façon à être en mesure de caractériser une réponse aversive des insectes.

Les individus testés se sont révélés capable d'associer le stimulus neutre (l'odeur) avec un renforcement négatif (la perspective d'être perturbé par le choc mécanique).

Dans ces deux expériences il s'agissait de démontrer la capacité des punaises à réaliser

une association entre un stimulus conditionnel clairement identifié et la possibilité d'obtenir une récompense ou une punition. Dans un second temps nous nous somme interrogés sur leur capacité à associer le complexe d'odeurs émanant d'un hôte vivant, avec la perspective de recevoir une punition. Les résultats obtenus au chapitre 3 révèlent que la préférence d'hôte peut elle aussi être biaisée, modifiée, sur la base de l'expérience individuelle. Le groupe pour lequel l'odeur de la caille a été appariée avec le choc mécanique, a montré une tendance (marginalement significative) à éviter la caille et à préférer l'odeur de rat lors du test. Ces résultats en particulier, suggèrent une influence de l'histoire de vie et de l'histoire évolutive des insectes. Ces punaises sont en effet historiquement associées aux oiseaux (Zeledon et Rabinovich, 1981) et l'élevage des individus testés était maintenu en alimentant les insectes sur des poules. Une association préalable a pu être établie entre l'alimentation régulière sur des volatiles et leur signature olfactive proche de celle des cailles, rendant plus difficile le conditionnement aversif de la réponse à ce bouquet d'odeurs.

Il parait intéressant de souligner qu'au cours des trois expériences réalisées (conditionnements appétitif, aversif et conditionnement de la réponse à des signaux complexes), les individus entrainés ont été capable d'utiliser une information dans un contexte particulier (dispositif de test) alors qu'elle avait été acquise dans un contexte différent (dispositif d'entrainement). Cela signifierait qu'une plus grande importance serait accordée au sens de l'information plutôt qu'au contexte spatial dans lequel l'information a été acquise. Nous verrons plus loin qu'il n'en est pas de même lorsqu'il s'agit du contexte temporel.

Pour résumer, selon leur expérience individuelle, les insectes sont donc capables d'attribuer une signification positive (conditionnement appétitif) ou négative (conditionnement aversif) à une même odeur neutre. Nous avons également pu démontrer que le choix de l'hôte pouvait être biaisé par l'expérience des individus. Cela signifie pour les punaises hématophages, être capables d'apprendre à associer une odeur de l'hôte avec soit la possibilité d'obtenir un repas sanguin, soit la possibilité d'être exposé au comportement défensif de l'hôte. Ces résultats ont un impact direct sur la *fitness* de l'insecte qui va pouvoir maximiser ses chances de se procurer de la nourriture et minimiser les risques associés à la récupéra-

tion de l'aliment. Une conséquence épidémiologique indirecte peut également être identifiée. En effet, comme l'on estimé Woolhouse *et al.* (1997), en moyenne 20 % d'une population d'hôte contribue à 80 % de la transmission nette d'un pathogène. En d'autres termes, l'hétérogénéité de la distribution des vecteurs au sein des populations d'hôtes tend à créer des "hot spots" (points chauds) et "cold spots" (points froids) de transmission. L'hétérogénéité du comportement de piqûre a donc une importance pratique considérable, en particulier lorsqu'il s'agit de mettre en place des stratégies de contrôle d'une maladie transmise par un insecte vecteur (Kelly et Thompson, 2000). Si le pattern de piqûre est prédictible, le contrôle des vecteurs peut être ciblé sur les points chauds ("hot-spots"), augmentant l'efficacité des stratégies mises en place. Il est fortement possible que l'apprentissage et la mémoire participent de manière importante à l'hétérogénéité observée dans la distribution des vecteurs au sein des populations d'hôtes, accentuant la préférence pour les hôtes les plus affaiblis et les moins défensifs.

Les résultats de cette première partie révèlent l'importance de la prise en compte des particularités biologiques du modèle étudié dans la mise en place d'études sur l'apprentissage et la mémoire. De même, le respect du cadre théorique et conceptuel imposé par l'étude des capacités cognitives des insectes, semble être un facteur clé pour une démonstration claire et irréfutable de ces dernières.

Conditionnement de la réponse d'extension du proboscis (PER).

Les résultats obtenus jusqu'à présent ont permis de mettre en évidence les capacités d'apprentissage des punaises hématophages *R. prolixus* et d'évaluer l'implication de ces processus cognitifs dans la réponse des insectes aux signaux des hôtes. Néanmoins, l'adaptation de procédures de conditionnement classique aux particularités biologiques des hématophages impliquent de réaliser un effort de contrôle important afin de placer les insectes testés dans des conditions motivationnelles et expérimentales favorables à la réalisation du processus d'apprentissage.

De telles contraintes expérimentales ne sont que peu compatibles avec l'analyse approfondie des mécanismes qui sous-tendent l'apprentissage et la mémoire. Pour cette raison, nous avons cherché à mettre en place un paradigme plus simple, permettant de réaliser des expériences de conditionnement en série et d'obtenir des résultats fiables et facilement reproductibles. La procédure de conditionnement de la PER, développée chez la drosophile et l'abeille, était adaptable à notre modèle d'étude et présentait de nombreux avantages. Parmi ces derniers, la possibilité de réaliser un conditionnement standardisé et largement validé chez les modèles classiques, offrait de nouvelles perspectives concernant la caractérisation des formes d'apprentissage dont est capable *R. prolixus*.

Contrairement aux mouches ou aux abeilles, la PER des triatomines ne peut pas être déclenchée par la présentation d'une solution sucrée ou une goute de sang. Les punaises sont strictement solénophages [1] et la chaleur est le seul stimulus nécessaire et suffisant pour déclencher la PER.

Nous avons donc utilisé un élément Peltier pour délivrer un stimulus thermique (35℃) et déclencher la PER. Après avoir démontré la possibilité d'habituer et déshabituer cette réponse, nous avons appariée la réponse des insectes avec la présentation d'un choc thermique (i.e. l'augmentation rapide de la température de l'élément Peltier, à 50℃). Cette procédure de conditionnement aversif de la PER a conduit les individus à cesser de répondre à la chaleur plus rapidement que par simple habituation et nous a permis d'estimer la durée de rétention de l'information mémorisée à 72h. En revanche, le fait que le stimulus inconditionnel (la stimulation appétitive à 35℃) et le renforcement négatif soient délivrés par le même dispositif, ne permet pas de contrôler l'effet des stimuli présentés seuls ou en l'absence de contingence. De même, le choc étant délivré si et seulement si le proboscis de l'individu est étendu et en contact avec la surface de l'élément Peltier, la réalisation d'un contrôle de type "*yoked-control*" (c'est-à-dire soumis à la même quantité de stimulation mais en l'absence de corrélation entre la réponse comportementale de l'insecte et l'occurrence du renforcement), ne serait pas rigoureuse. En effet la présence d'un objet à

1. Ces insectes puisent le sang directement dans les capillaires sanguins

50°C placé face à l'insecte n'a pas les mêmes conséquences qu'un choc délivré à l'extrémité du proboscis. La visualisation des deux situations à l'aide d'une caméra thermographique permet de rendre compte des différences (observations non présentées).

Réaliser une expérience de conditionnement olfactif de la PER permettrait de s'affranchir de ces contraintes liées au dispositif expérimental. Odeurs, chaleur et renforcement n'étant pas délivrés par le même dispositif, il serait possible de tester l'effet de chaque stimulus agissant seul et l'effet des stimuli en l'absence de contingence. Ce type de conditionnement implique de franchir plusieurs obstacles. Nous avons montré qu'il est possible de déclencher la PER en délivrant un stimulus thermique à l'insecte. De même les expériences de conditionnement olfactif ont apporté des solutions simples pour la présentation d'odeurs. La difficulté inhérente au conditionnement olfactif de la PER réside dans la présentation du renforcement positif. Pour ces insectes le seul renforcement envisageable est l'absorption d'une quantité limitée (pour ne pas modifier l'état motivationnel) de sang, éventuellement, son remplacement par solution saline contenant des phagostimulants, une procédure qui reste à tester. Dans les deux cas, la solution délivrée doit être chaude, c'est-à-dire à une température perçue comme appétitive par les punaises. Il s'agirait donc de délivrer l'odeur (CS), déclencher la PER (réponse inconditionnelle, RI) en augmentant la température du Peltier (US), et renforcer l'appariement en permettant aux insectes d'ingérer une petite quantité de fluide chaud (renforcement positif). Afin de tester l'apprentissage il faudrait ensuite évaluer si la présentation de l'odeur seule (CS) suffit à déclencher la PER (réponse conditionnée, RC). Ces essais constitueront probablement les prochaines étapes dans l'étude des possibilités de conditionnement de la PER chez les triatomines. Le caractère strictement solénophage des triatomines impose, cependant, une contrainte importante pour la mise à point d'un protocole expérimental de ce type.

Le paradigme expérimental tel que nous l'avons développé permet néanmoins d'identifier des facteurs modulateurs de l'apprentissage. En particulier, nous avons démontré que la capacité d'apprendre, et non la capacité de faire appel à l'information mémorisée, est sous le contrôle d'une horloge endogène, révélant ainsi un rythme véritablement circadien.

Si nous avions vu dans la première partie de ce travail que le sens de l'information acquise primait sur le contexte spatial, il en va autrement pour le contexte temporel.

Ces résultats représentent la deuxième démonstration seulement d'un tel phénomène chez les insectes en général (Decker *et al.*, 2007). L'apprentissage étant un processus énergétiquement couteux et pertinent seulement si les souvenirs sont utilisés dans le contexte temporel dans lequel ils ont été formés, il pourrait s'agir d'un phénomène largement répandu chez les insectes.

De manière générale, concernant l'étude des capacités d'apprentissage, il parait donc nécessaire de souligner le caractère déterminant du contexte temporel, pour la mise en évidence de ces capacités et l'analyse des mécanismes sous-jacents. La synchronisation des individus et la prise en compte du moment du jour subjectif se révèlent en effet être les clés d'une analyse pertinente de ces processus cognitifs.

Conclusions et perspectives.

La rareté des études proposant des démonstrations claires des capacités d'apprentissage chez les insectes vecteurs de maladies, semble donc être le résultat d'un manque de contrôle de différents paramètres. Les études de terrain ne permettant pas de contrôler tous les facteurs pouvant moduler le comportement des insectes, l'expérimentation contrôlée, au laboratoire parait essentielle pour une démonstration et une caractérisation claire, bien qu'elle ne soit pas suffisante pour appréhender l'ampleur des bénéfices apportés par l'apprentissage. Afin d'optimiser les chances de placer l'insecte dans une situation propice à révéler ses capacités d'apprentissage, il ressort des résultats obtenus dans ce travail qu'il est primordial de tenir compte des particularités biologiques du modèle, du moment du jour subjectif et du cadre théorique imposé par l'étude de l'apprentissage.

De façon plus générale, il ressort de l'étude de la littérature que ce sujet souffre d'un manque d'interactions entre deux communautés. D'un côté les épidémiologistes et les entomologistes médicaux concentrent leur attention sur le vecteur, de l'autre les physiologistes

et les neurobiologistes étudiant les mécanismes se trouvant à l'origine des comportements observés. Ce type de travail sur l'étude des capacités d'apprentissage implique, au contraire, d'entretenir des interactions avec les deux mondes. En effet, de telles études sont impensables sans tenir compte des connaissances accumulées par les deux communautés.

Pour terminer, les travaux présentés ici, ne constituent qu'un premier pas vers la caractérisation et la compréhension de l'apprentissage et de la mémoire chez les insectes vecteurs de maladies. Ils apportent l'adaptation de paradigmes expérimentaux largement validés chez les modèles classiques (conditionnement olfactif, conditionnement de la PER), confirment l'influence de l'apprentissage dans le choix des hôtes et soulignent l'importance de facteurs motivationnels et temporels dans la mise en évidence de ce phénomène. Pris dans son ensemble, ce travail de thèse pose un grand nombre de questions. Des plus fondamentales : Quels mécanismes soutiennent la formation de la mémoire observée ? La synthèse de protéines est-elle impliquée dans ces processus ? Les insectes hématophages sont ils capables d'utiliser à l'état adulte des informations acquises pendant la vie larvaire ? A quel point l'apprentissage et la mémoire sont développés chez les vecteurs de maladies ? etc.

... aux plus appliquées : L'apprentissage peut-il être utilisé comme outil afin de répondre à des questions biologiques (plus petite différence discriminable, etc.) ? Quel est l'impact de l'apprentissage sur les stratégies de contrôle ? La manipulation du comportement vectoriel est elle possible par le conditionnement des réponses aux signaux naturels des hôtes ?

Pour résumer, ce travail apporte à la fois des pistes à suivre pour l'étude de l'apprentissage et permet d'identifier des cibles potentielles pour le contrôle des vecteurs et par conséquent, la lutte contre les maladies qu'ils transmettent.

Annexe

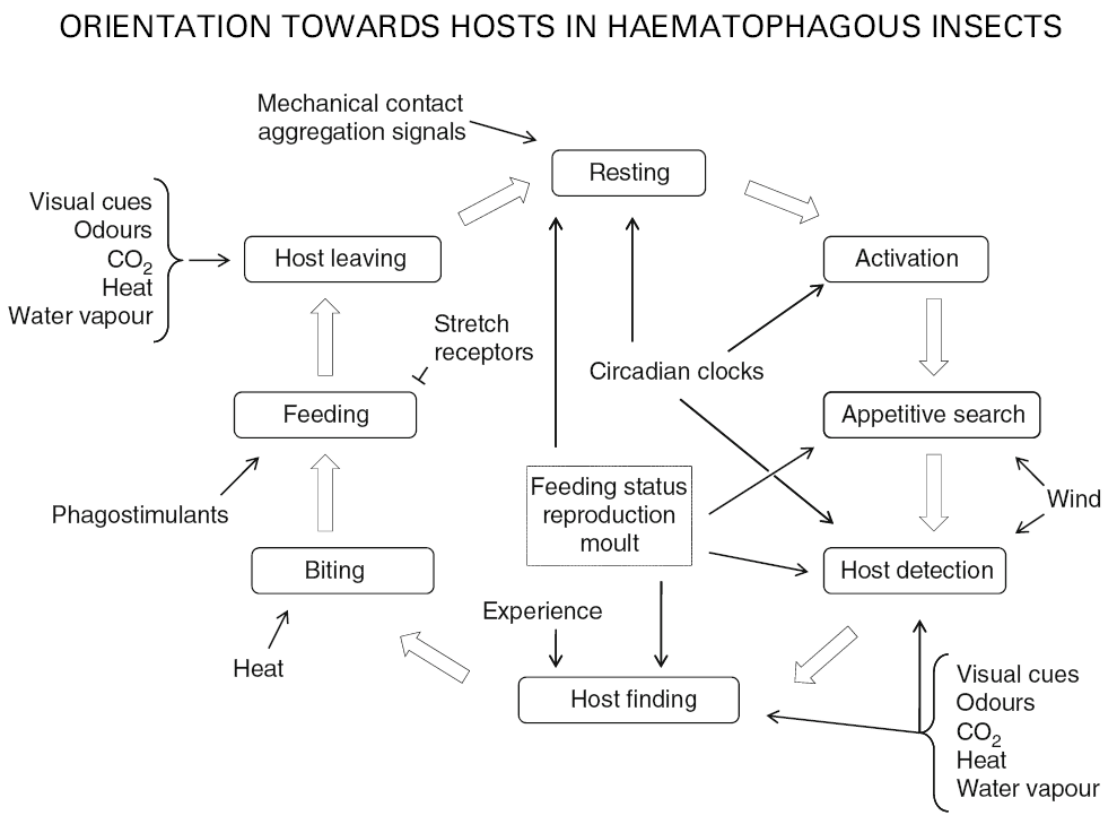

FIGURE 6 – Facteurs modulant l'hématophagie chez les insectes vecteurs de maladies. Les facteurs exogènes sont représentés à l'extérieur du cercle et les facteurs endogènes sont présentés à l'intérieur du cercle. L'insecte s'acive à un moment particulier de la journée, sous l'influence d'horloges internes. Si son état reproductif est approprié (affamé, pas d'activité reproductrice), il se met à la recherche de signaux appétitifs, en utilisant la direction du vent comme direction de référence. la présence de signaux associés aux hôtes permet à l'insecte de détecter sa source de nourriture et de la localiser. La sensibilité et la réponse à ces signaux sont modulées par des facteurs endogènes (*state-dependency*). L'expérience de l'individu conditionne l'approche finale de l'hôte ou la sélection d'hôte si plusieurs d'entre eux sont présents. Une fois sur l'hôte, l'insecte doit choisir l'endroit le plus approprié pour piquer, guidé par des gradients de température sur la peau, indiquant le degré d'irrigation et la proximité de vaisseaux sanguins. Lorsque de la nourriture potentielle est trouvée, les chémorecepteurs situés dans le canal alimentaire vont tester le liquide et rechercher la présense de phagostimulants. l'insecte se nourrit ensuite jusqu'à ce que ses propriocepteurs mécaniques indique que le tube digestif est rempli de sang. Les pièces buccales se détachent alors de l'hôte et l'insecte s'éloigne de l'hôte. Finalement, l'insecte retourne s'abriter dans son refuge ou dans un endroit abrité en s'aidant de signaux spécifiques associés aux refuges (phéromones d'aggrégation, contact physique avec le substrat(thigmotaxie). (D'après Lazzari, 2009)

Bibliographie

Abramson, C., Romero, E., Frasca, J., Fehr, R., Lizano, E. & Aldana, E. 2005. Psychology of learning: A new approach to study behavior of *Rhodnius prolixus* stal under laboratory conditions. *Psychological Reports* **97**: 721–731.

Acree, F., Turner, R., Gouck, H., Beroza, M. & Smith, N. 1968. L-lactic acid - A mosquito attractant isolated from humans. *Science* **161**: 1346–1347.

Adams, M., Celniker, S., Holt, R., Evans, C., Gocayne, J., Amanatides, P. et al. 2000. The genome sequence of *Drosophila melanogaster*. *Science* **287**: 2185–2195.

Aldana, E., Abramson, C.I., Lizano, E., Vegas, R. & Sulbaran-Romero, E. 2008. Learning and orientation to odor in the bug *Rhodnius prolixus* Stal 1859 under laboratory conditions. *Parasitology Research* **103**: 587–594.

Alexander, B., Lopes de Carvalho, R., McCallum, H. & Pereira, M. 2002. Role of the domestic chicken (*Gallus gallus*) in the epidemiology of urban visceral leishmaniasis in brazil. *Emerging Infectious Diseases* **8**: 1480–1485.

Alloway, T. 1972. Learning and memory in insects. *Annual Review of Entomology* **17**: 43–56.

Alonso, W., Wyatt, T. & Kelly, D. 2003. Are vectors able to learn about their hosts? A case study with *Aedes aegypti* mosquitoes. *Memorias do Instituto Oswaldo Cruz* **98**: 665–672.

Alonso, W.J. & Schuck-Paim, C. 2006. The "ghosts" that pester studies on learning in mosquitoes: guidelines to chase them off. *Medical and Veterinary Entomology* **20**:

157–165.

Ampleford, E. & Steel, C. 1982. Circadian control of ecysis in *Rhodnius prolixus* (Hemiptera). *Journal of Comparative Physiology* **147**: 281–286.

Aschoff, J. 1989. Temporal orientation: circadian clocks in animals and humans. *Animal Behaviour* **37**: 881–896.

Ayestaran, A., Giurfa, M. & de Brito Sanchez, M. 2010. Toxic but drank: gustatory aversive compounds induce post-ingestional malaise in harnessed honeybees. *PLoS One* **5**: e15000.

Barron, A. & Corbet, S. 1999. Preimaginal conditioning in *Drosophila* revisited. *Animal Behaviour* **58**: 621–628.

Barrozo, R. & Lazzari, C. 2004a. Orientation behaviour of the blood-sucking bug *Triatoma infestans* to short-chain fatty acids: Synergistic effect of L-lactic acid and carbon dioxide. *Chemical Senses* **29**: 833–841.

Barrozo, R. & Lazzari, C. 2004b. The response of the blood-sucking bug *Triatoma infestans* to carbon dioxide and other host odours. *Chemical Senses* **29**: 319–329.

Barrozo, R., Manrique, G. & Lazzari, C. 2003. The role of water vapour in the orientation behaviour of the blood-sucking bug *Triatoma infestans* (Hemiptera, Reduviidae). *Journal of Insect Physiology* **49**: 315–321.

Barrozo, R., Minoli, S. & Lazzari, C. 2004. Circadian rhythm of behavioural responsiveness to carbon dioxide in the blood-sucking bug *Triatoma infestans* (Heteroptera : Reduviidae). *Journal of Insect Physiology* **50**: 249–254.

Baxter, C. 1957. Habituation of the reach to puffs of air. *Anatomical record* **15**: 704–712.

Bernier, U., Booth, M. & Yost, R. 1999. Analysis of human skin emanations by gas chromatography mass spectrometry. 1. Thermal desorption of attractants for the yellow fever mosquito (*Aedes aegypti*) from handled glass beads. *Analytical Chemistry* **71**: 1–7.

Bernier, U., Kline, D., Barnard, D., Schreck, C. & Yost, R. 2000. Analysis of human skin emanations by gas chromatography/mass spectrometry. 2. Identification of

volatile compounds that are candidate attractants for the yellow fever mosquito (*Aedes aegypti*). *Analytical Chemistry* **72**: 747–756.

Bitterman, M. 1996. Comparative analysis of learning in honeybees. *Animal Learning & Behavior* **24**: 123–141.

Bitterman, M., Menzel, R., Fietz, A. & Schafer, S. 1983. Classical-conditioning of proboscis extension in honeybees (*Apis mellifera*). *Journal of Comparative Psychology* **97**: 107–119.

Bodin, A., Barrozo, R.B., Couton, L. & Lazzari, C.R. 2008. Temporal modulation and adaptive control of the behavioural response to odours in *Rhodnius prolixus*. *Journal of Insect Physiology* **54**: 1343–1348.

Bodin, A., Vinauger, C. & Lazzari, C.R. 2009a. Behavioural and physiological state dependency of host seeking in the blood-sucking insect *Rhodnius prolixus*. *Journal of Experimental Biology* **212**: 2386–2393.

Bodin, A., Vinauger, C. & Lazzari, C.R. 2009b. State-dependency of host-seeking in *Rhodnius prolixus*: The post-ecdysis time. *Journal of Insect Physiology* **55**: 574–579.

Bouyer, J., Pruvot, M., Bengaly, Z., Guerin, P. & Lancelot, R. 2007. Learning influences host choice in tsetse. *Biology Letters* **3**: 113–117.

Braks, M., Anderson, R. & Knols, B. 1999. Infochemicals in mosquito host selection: Human skin microflora and *Plasmodium parasites*. *Parasitology Today* **15**: 409–413.

Braun, G. & Bicker, G. 1992. Habituation of an appetitive reflex in the honeybee. *Journal of Neurophysiology* **67**: 588–598.

Brembs, B. 1996. *Operant and classical conditioning of Drosophila at the flight simulator*. Ph.D. thesis, Julius-Maximilians Universitat Wurzburg Fakultat fur Biologie.

Bromenshenk, J., Henderson, C., Seccomb, R., Rice, S., Etter, R., Bender, S., Rodacy, P., Shaw, J., Seldomridge, N., Spangler, L. & Wilson, J. 2003. Can honey bees assist in area reduction and landmine detection? *Research, Development and Technology in Mine Action* **7**: n.a.

Bush, R. & Mosteller, F. 1955. *Stochastic models for learning.* Wiley, Oxford, p. 365.

Campbell-Lendrum, D., Brandao-Filho, S., Ready, P. & Davies, C.R. 1999. Host and/or site loyalty of *Lutzomyia whitmani* (diptera: Psychodidae) in brazil. *Medical and Veterinary Entomology* **13**: 209–211.

Carcaud, J., Roussel, E., Giurfa, M. & Sandoz, J.C. 2009. Odour aversion after olfactory conditioning of the sting extension reflex in honeybees. *Journal of Experimental Biology* **212**: 620–626.

Chabaud, M., Devaud, J., Pham-Delègue, M., Preat, T. & Kaiser, L. 2006. Olfactory conditioning of proboscis activity in *Drosophila melanogaster*. *Journal of Comparative Physiology A-Neuroethology Sensory Neural and Behavioral Physiology* **192**: 1335–1348.

Châline, N., Sandoz, J., Martin, S., Ratnieks, F. & Jones, G. 2005. Learning and discrimination of individual cuticular hydrocarbons by honeybees (*Apis mellifera*). *Chemical Senses* **30**: 327–335.

Charlwood, J., Graves, P. & Marshall, T. 1988. Evidence for a memorized home range in *Anopheles farauti* females from Papua-New-Guinea. *Medical and Veterinary Entomology* **2**: 101–108.

Chaudhury, D., Wang, L. & Colwell, C. 2005. Circadian regulation of hippocampal long-term potentiation. *Journal of Biological Rhythms* **20**: 225–236.

Clements, A. 1999. *The Biology of Mosquitoes: Sensory Reception and Behaviour (vol.2)*, chap. Flight, pp. 287–332. CABI Publishing.

Cohen, M. & Nagel, E. 1934. *Logic and Scientific Method.* Harcourt, New York, p. 467.

Collado, G. & Castella, C. 1934. Un caso interesante de emigración orientada del *A. maculipennis* durante la epoca invernal. *Revista de Sanidad e Higiene Pública* **6**: 29–33.

Colwill, R., Absher, R. & ML, R. 1988. Conditional discrimination learning in *Aplysia californica*. *Journal of Neuroscience* **8**: 4440–4444.

Constantinou, C. 1984. Circadian rhythm of oviposition in the blood sucking bugs, *Triatoma phyllosoma*, *Triatoma infestans* and *Panstrongylus megistus* (Hemiptera, Reduviidae). *Journal of Interdisciplinary Cycle Research* **15**: 203–211.

Cork, A. & Park, K. 1996. Identification of electrophysiologically-active compounds for the malaria mosquito, *Anopheles gambiae*, in human sweat extracts. *Medical and Veterinary Entomology* **10**: 269–276.

Corning, W.C., Dyal, J.A. & Willows, A. 1973a. *Invertebrate learning: I. Protozoans through annelids.* Plenum Press, New York, p. 296.

Corning, W.C., Dyal, J.A. & Willows, A. 1973b. *Invertebrate learning: II. Arthropods and Gasteropod Mollusks.* Plenum Press, New York, p. 284.

Costa, A., Ricard, I., Davison, A.C. & Turlings, T.C.J. 2010. Effects of rewarding and unrewarding experiences on the response to host-induced plant odors of the generalist parasitoid *Cotesia marginiventris* (Hymenoptera: Braconidae). *Journal of Insect Behavior* **23**: 303–318.

Daly, K. & Smith, B. 2000. Associative olfactory learning in the moth *Manduca sexta*. *Journal of Experimental Biology* **203**: 2025–2038.

Day, J. & Edman, J. 1984. Mosquito engorgement on normally defensive hosts depends on host activity patterns. *Journal of Medical Entomology* **21**: 732–740.

de Brito Sanchez, M., Giurfa, M., de Paula Mota, T. & Gauthier, M. 2005. Electrophysiological and behavioural characterization of gustatory responses to antennal "bitter" taste in honeybees. *European Journal of Neuroscience* **22**: 3161–3170.

de Jianne, D., Mc Guire, T. & Pruzan-Hotchkiss, A. 1985. Conditioned suppression of proboscis extension in *Drosophila melanogaster*. *Journal of Comparative Psychology* **99**: 74–80.

Decker, S., McConnaughey, S. & Page, T. 2007. Circadian regulation of insect olfactory learning. *Proceedings of the National Academy of Sciences of the United States of America* **104**: 15905–15910.

Deisig, N., Lachnit, H. & M, G. 2001. The effect of similarity between elemental stimuli and compounds in olfactory patterning discriminations. *Learning & Memory* **9**: 112–121.

Dias, J., Silveira, A. & Schofield, C. 2002. The impact of Chagas disease control in Latin America - A review. *Memorias do Instituto Oswaldo Cruz* **97**: 603–612.

Domjan, M. 1998. *The principles of learning and behavior*. Brooks/Cole, Pacific Grove, CA, p. 663.

Dubnau, J. & Tully, T. 1998. Gene discovery in Drosophila: New insights for learning and memory. *Annual Review of Neuroscience* **21**: 407–444.

Dukas, R. 2008. Evolutionary biology of insect learning. *Annual Review of Entomology* **53**: 145–160.

Dukas, R. & Bernays, E. 2000. Learning improves growth rate in grasshoppers. *Proceedings of the National Academy of Sciences of the United States of America* **97**: 2637–2640.

Dukas, R. & Duan, J. 2000. Potential fitness consequences of associative learning in a parasitoid wasp. *Behavioral Ecology* **11**: 536–543.

Dye, C. 1992. The analysis of parasite transmission by bloodsucking insects. *Annual Review of Entomology* **37**: 1–19.

Dye, C. & Hasibeder, G. 1986. Population-dynamics of mosquito-borne disease - Effects of flies which bite some people more frequently than others. *Transaction of the Royal Society of Tropical Medicine and Hygiene* **80**: 69–77.

Edman, J., Day, J. & Walker, E. 1984. Field confirmation of laboratory observations on the differential antimosquito behavior of herons. *The Condor* **86**: 91–92.

Edman, J. & Scott, T. 1987. Host defensive behavior and the feeding success of mosquitoes. *Insect Science and its Application* **8**: 617–622.

Edman, J.D., A., W.L. & Kale, H.W. 1972. Effect of mosquito density on the interrelationship of host behavior and mosquito feeding success. *American Journal of Tropical Medicine and Hygiene* **21**: 487–491.

Eiras, A. & Jepson, P. 1991. Host location by *Aedes aegypti* (Diptera, Culicidae) - a wind-tunnel study of chemical cues. *Bulletin of Entomological Research* **81**: 151–160.

Eisenstein, E. 1967. *The Neurosciences: A Study Program*, chap. The use of invertebrate systems for studies on the basis of learning and memory, pp. 653–665. Rockefeller University Press.

Erber, J., Pribbenow, B., Grandy, K. & Kierzek, S. 1997. Tactile motor learning in the antennal system of the honeybee (*Apis mellifera*). *Journal of Comparative Physiology A-Neuroethology Sensory Neural and Behavioral Physiology* **181**: 355–365.

Fernandez, R., Lyons, L., Levenson, J., Khabour, O. & Eskin, A. 2003. Circadian modulation of long-term sensitization in *Aplysia*. *Proceedings of the National Academy of Sciences of the United States of America* **100**: 14415–14420.

Ferreira, R.A., Lazzari, C.R., Lorenzo, M.G. & Pereira, M.H. 2007. Do haematophagous bugs assess skin surface temperature to detect blood vessels? *PLoS One* **2**: e392.

Flores, G. & Lazzari, C. 1996. The role of the antennae in *Triatoma infestans*: Orientation towards thermal sources. *Journal of Insect Physiology* **42**: 433–440.

Fresquet, N. 1999. Effects of aging on the acquisition and extinction of excitatory conditioning in *Drosophila melanogaster*. *Physiology & Behavior* **67**: 205–211.

Friend, W. & Smith, J. 1977. Factors affecting feeding by bloodsucking insects. *Annual Review of Entomology* **22**: 309–331.

Frings, H. 1941. The loci of olfactory end-organs in the blow-fly *Cynomyia cadaverina* desvoidy. *Journal of Experimental Zoology* **88**: 65–93.

Frings, H. 1944. The loci of olfactory end-organs in the honey-bee, *Apis mellifera* linn. *Journal of Experimental Zoology* **97**: 123–134.

Galizia, C. & Menzel, R. 2000. Odour perception in honeybees: coding information in glomerular patterns. *Current Opinion in Neurobiology* **10**: 504–510.

Geier, M., Sass, H. & Boeckh, J. 1996. A search for components in human body odour that attract females of *Aedes aegypti*. In: *Olfaction in mosquito-host interactions* (Bock, GR and Cardew, G, ed), vol. 200 of *CIBA Foundation Symposia*, pp. 132–148.

Giurfa, M. 2003. Cognitive neuroethology: dissecting non-elemental learning in a honeybee brain. *Current Opinion in Neurobiology* **13**: 726–735.

Giurfa, M. 2007. Behavioral and neural analysis of associative learning in the honeybee: a taste from the magic well. *Journal of Comparative Physiology A-Neuroethology Sensory Neural and Behavioral Physiology* **193**: 801–824.

Giurfa, M., Eichmann, B. & Menzel, R. 1996. Symmetry perception in an insect. *Nature* **382**: 458–461.

Giurfa, M. & Malun, D. 2004. Associative mechanosensory conditioning of the proboscis extension reflex in honeybees. *Learning & Memory* **11**: 294–302.

Giurfa, M. & Menzel, R. 2003. Cognitive architecture of a mini-brain. In: *Adaptivity and learning - An interdisciplinary debate*, pp. 23–48. Workshop on Cognition, Learning and Adaptivity, Bielefelf, Germany, Sep, 2000.

Giurfa, M., Zhang, S., Jennett, A., Menzel, R. & Srinivasan, M.V. 2001. The concepts of sameness and differences in an insect. *Nature* **410**: 930–933.

Grabowski, C.T. & Dethier, V.G. 1954. The structure of the tarsal chemoreceptors of the blowfly, *Phormia regina* meigen. *Journal of Morphology* **94**: 1–19.

Guerenstein, P., Lorenzo, M., Nunez, J. & Lazzari, C. 1995. Bakers-yeast, an attractant for baiting traps for Chagas-disease vectors. *Experientia* **51**: 834–837.

Guerenstein, P.G. & Lazzari, C.R. 2009. Host-seeking: How triatomines acquire and make use of information to find blood. *Acta Tropica* **110**: 148–158.

Habib, M. 2007. Controlled biological and biomimetic systems for landmine detection. *Biosensors and Bioelectronics* **23**: 1–18.

Hammer, M. 1993. An identified neuron mediates the unconditioned stimulus in associative olfactory learning in honeybees. *Nature* **366**: 69–63.

Hasibeder, G. & Dye, C. 1988. Population-dynamics of mosquito-borne disease - Persistence in a completely heterogeneous environment. *Theoretical Population Biology* **33**: 31–53.

Hasibeder, G., Dye, C. & Carpenter, J. 1992. Mathematical-modeling and theory for estimating the basic reproduction number of canine leishmaniasis. *Parasitology* **105**: 43–53.

Hayes, W.P. & Liu, Y.S. 1947. Tarsal chemoreceptors of the housefly and their possible relation to ddt toxicity. *Annals of the Entomological Society of America* **40**: 401–416.

Hebb, D. 1949. *The organization of behavior; a neuropsychological theory.* Wiley, New York.

Heisenberg, M. & Wolf, R. 1991. Basic organization of operant behavior as revealed in *Drosophila* flight orientation. *Journal of Comparative Physiology A-Neuroethology Sensory Neural and Behavioral Physiology* **169**: 699–705.

Hermitte, G., Pedreira, M., Tomsic, D. & Maldonado, H. 1999. Context shift and protein synthesis inhibition disrupt long-term habituation after spaced, but not massed, training in the crab *Chasmagnathus*. *Neurobiology of Learning and Memory* **71**: 34–49.

Hii, J., Chew, M., Sang, V., Munstermann, L., Tan, S., Panym, S. & Yasothornskikul, S. 1991. Population genetic analysis of host seeking and resting behaviors in the malaria vector, *Anopheles balabacensis* (diptera, culicidae). *Journal of Medical Entomology* **28**: 675–684.

Holliday, M. & Hirsch, J. 1986. Excitatory conditioning of individual *Drosophila melanogaster*. *Journal of Experimental Psychology: Animal Behavior Processes* **12**: 131–142.

Horridge, G. 1997. Vision of the honeybee *Apis mellifera* for patterns with one pair of equal orthogonal bars. *Journal of Insect Physiology* **43**: 741–748.

Hourcade, B., Muenz, T., Sandoz, J.C., Rossler, W. & Devaud, J.M. 2010. Long-term memory leads to synaptic reorganization in the mushroom bodies: a memory trace in the insect brain? *The Journal of Neuroscience* **30**: 6461–6465.

Hourcade, B., Perisse, E., Devaud, J.M. & Sandoz, J.C. 2009. Long-term memory shapes the primary olfactory center of an insect brain. *Learning & Memory* **16**: 607–615.

Jhumur, U.S., Doetterl, S. & Juergens, A. 2006. Naive and conditioned responses of *Culex pipiens pipiens* biotype molestus (Diptera : Culicidae) to flower odors. *Journal of Medical Entomology* **43**: 1164–1170.

Kale, H., Webber, L. & Edman, J. 1972. Effect of behavior and age of individual ciconiform birds on mosquito feeding success. *Mosquito News* **32**: 343–350.

Kaur, J., Lai, Y. & Giger, A. 2003. Learning and memory in the mosquito *Aedes aegypti* shown by conditioning against oviposition deterrence. *Medical and Veterinary Entomology* **17**: 457–460.

Kelly, D. 2001. Why are some people bitten more than others? *Trends in Parasitology* **17**: 578–581.

Kelly, D. & Dye, C. 1997. Pheromones, kairomones and the aggregation dynamics of the sandfly *Lutzomyia longipalpis*. *Animal Behaviour* **53**: 721–731.

Kelly, D., Mustafa, Z. & Dye, C. 1996. Density-dependent feeding success in a field population of the sandfly, *Lutzomyia longipalpis*. *Journal of Animal Ecology* **65**: 517–527.

Kelly, D. & Thompson, C. 2000. Epidemiology and optimal foraging: modelling the ideal free distribution of insect vectors. *Parasitology* **120**: 319–327.

Klowden, M. 1990. The endogenous regulation of mosquito reproductive behavior. *Experientia* **46**: 660–669.

Klowden, M. 1997. Endocrine aspects of mosquito reproduction. *Archives of Insect Biochemistry and Physiology* **35**: 491–512.

Klowden, M. & Blackmer, J. 1987. Humoral control of pre-oviposition behaviour in the mosquito, *Aedes aegypti*. *Journal of Insect Physiology* **33**: 689–692.

Klowden, M. & Briegel, H. 1994. Mosquito gonotrophic cycle and multiple feeding potential - Contrasts between *Anopheles* and *Aedes* (Diptera, Culicidae). *Journal of Medical Entomology* **31**: 618–622.

Klowden, M. & Lea, A. 1979. Effect of defensive host behavior on the blood meal size and feeding success of natural-populations of mosquitos (Diptera, Culicidae). *Journal of Medical Entomology* **15**: 514–517.

Konorski, J. 1948. *Conditioned reflexes and neuron organization*. Cambridge University Press, London, p. 267.

Kuwabara, M. 1957. Bildung des bedingten reflexes von pavlovs typus bei der honigbiene, *Apis mellifera*. *Journal of the Faculty of Science, Hokkaido University, Zoology* **13**: 458–464.

Lazzari, C. 1992. Circadian organization of locomotion activity in the hematophagous bug *Triatoma infestans*. *Journal of Insect Physiology* **38**: 895–903.

Lazzari, C. 2009. Orientation towards hosts in haematophagous insects: An integrative perspective. *Advances in Insect Physiology* **37**: 1–58.

Lazzari, C. & Núñez, J. 1989. Blood temperature and feeding-behavior in *Triatoma infestans* (Heteroptera, Reduviidae). *Entomologia Generalis* **14**: 183–188.

Lazzari, C. & Varjú, D. 1990. Visual lateral fixation and tracking in the hematophagous bug *Triatoma infestans*. *Journal of Comparative Physiology A-Sensory Neural and Behavioral Physiology* **167**: 527–531.

Lazzari, C.R. & Lorenzo, M.G. 2009. Exploiting triatomine behaviour: alternative perspectives for their control. *Memorias do Instituto Oswaldo Cruz* **104**: 65–70.

Lehane, M.J. 2005. *The biology of blood-sucking in insects*. Cambridge University Press, New York.

Livingston, R. 1966. Brain mechanisms in conditioning and learning. *N R P Bull.* **4**: 235–347.

Lorenz, K. 1981. *The foundations of ethology.* Springer-Verlag, New-York, p. 380.

Lorenzo, M., Reisenman, C. & Lazzari, C. 1998. *Triatoma infestans* can be captured under natural climatic conditions using yeast-baited traps. *Acta Tropica* **70**: 277–284.

Lorenzo-Figueiras, A. & Lazzari, C. 1998. Aggregation behaviour and interspecific responses in three species of triatominae. *Memorias do Instituto Oswaldo Cruz* **93**: 133–137.

Lucchetta, P., Bernstein, C., Thery, M., Lazzari, C. & Desouhant, E. 2008. Foraging and associative learning of visual signals in a parasitic wasp. *Animal Cognition* **11**: 525–533.

Lyons, L., Rawashdeh, O. & Eskin, A. 2006. Non-ocular circadian oscillators and photoreceptors modulate long term memory formation in *Aplysia*. *Journal of Biological Rhythms* **21**: 245–255.

Malun, D., Giurfa, M., Galizia, G., Plath, N., Brandt, R., Gerber, B. & Eisermann, B. 2002. Hydroxyurea-induced partial mushroom body ablation does not affect acquisition and retention of olfactory differential conditioning in honeybees. *Journal of Neurobiology* **53**: 343–360.

Margulies, C., Tully, T. & Dubnau, J. 2005. Deconstructing memory in *Drosophila*. *Current Biology* **15**: R700–R713.

Mauelshagen, J. 1993. Neural correlates of olfactory learning in an identified neuron in the honey bee brain. *Journal of Neurophysiology* **69**: 609–625.

McCall, P. & Eaton, G. 2001. Olfactory memory in the mosquito *Culex quinquefasciatus*. *Medical and Veterinary Entomology* **15**: 197–203.

McCall, P. & Kelly, D. 2002. Learning and memory in disease vectors. *Trends in Parasitology* **18**: 429–433.

McCall, P., Mosha, F., Njunwa, K. & Sherlock, K. 2001. Evidence for memorized site-fidelity in *Anopheles arabiensis*. *Transaction of the Royal Society of Tropical Medicine and Hygiene* **95**: 587–590.

Menzel, R. 1999. Memory dynamics in the honeybee. *Journal of Comparative Physiology A-Sensory Neural and Behavioral Physiology* **185**: 323–340.

Menzel, R. 2001. Searching for the memory trace in a mini-brain, the honeybee. *Learning & Memory* **8**: 53–62.

Menzel, R., Brembs, B. & Giurfa, M. 2007. Cognition in Invertebrates. In: *Evolution of nervous systems in invertebrates, vol. II Evolution of nervous systems* (Oxford: Academic Press., ed), pp. 403–422.

Menzel, R. & Giurfa, M. 2001. Cognitive architecture of a mini-brain: the honeybee. *Trends in Cognitive Sciences* **5**: 62–71.

Menzel, R. & Muller, U. 1996. Learning and memory in honeybees: from behavior to neural substrates. *Annual Review of Neuroscience* **19**: 379–404.

Mery, F. 2006. Evolution of behavioral plasticity in *Drosophila*: Costs and benefits of learning and memory. *Chemical Senses* **31**: 299. 17th Congress of the European-Chemoreception-Research-Organization (ECRO), Granada, Spain, Sep, 2006.

Mery, F. 2007. Experimental evolution of learning ability in *Drosophila melanogaster*: Benefits and fitness costs of learning. *Comparative Biochemistry and Physiology A-Molecular & Integrative Physiology* **146**: 15.

Mery, F. & Kawecki, T. 2002. Experimental evolution of learning ability in fruit flies. *Proceedings of the National Academy of Sciences of the United States of America* **99**: 4274–4279.

Mery, F. & Kawecki, T. 2005. A cost of long-term memory in *Drosophila*. *Science* **308**: 1148.

Mery, F., Pont, J., Preat, T. & Kawecki, T.J. 2007. Experimental evolution of olfactory memory in *Drosophila melanogaster*. *Physiological and Biochemical Zoology* **80**: 399–405.

Mikulas, W. 1974. *Concepts in Learning.* W. B. Saunders Company, p. 235.

Miller, N. 1967. *The Neurosciences: A Study Program*, chap. Certain facts of learning relevant to the search for its physical basis, pp. 643–652. Rockefeller University Press, New York.

Mwandawiro, C., Boots, M., Tuno, N., Suwonkerd, W., Tsuda, Y. & Takagi, M. 2000. Heterogeneity in the host preference of Japanese encephalitis vectors in Chiang Mai, northern Thailand. *Transaction of the Royal Society of Tropical Medicine and Hygiene* **94**: 238–242.

Núñez, J., Insausti, T. & Lazzari, C. 1996. Rearing of *Triatoma infestans* Klug (Het, Reduviidae) in the absence of a live host. 2. Egg yolk as a diet supplement. *Journal of Applied Entomology - Zeitschrift fur Angewandte Entomologie* **120**: 541–547.

Núñez, J. & Lazzari, C. 1990. Rearing of *Triatoma infestans* Klug (Het, Reduviidae) in the absence of a live host. 1. Some factors affecting the artificial feeding. *Journal of Applied Entomology - Zeitschrift fur Angewandte Entomologie* **109**: 87–92.

Okasha, A. 1968a. Effects of sub-lethal high temperature on an insect, *Rhodnius prolixus* (stal). i. induction of delayed moulting and defects. *Journal of Experimental Biology* **48**: 455–463.

Okasha, A. 1968b. Effects of sub-lethal high temperature on an insect, *Rhodnius prolixus* (stal). iii. metabolic changes and their bearing on the cessation and delay of moulting. *Journal of Experimental Biology* **48**: 475–486.

Papaj, D.R. & Lewis, A.C. 1993. *Insect learning: Ecological and evolutionary perspectives.* Chapman & Hall, New York, p. 398.

Pearce, J. 2008. *Animal Learning & cognition.* Psychology Press, Hove, East Sussex, p. 420.

Pierce, B. 2004. *Genetics: A Conceptual Approach.* W. H. Freeman. (2nd ed.), p. 832.

R 2010. R: A language and environment for statistical computing.

Raine, N.E. & Chittka, L. 2008. The correlation of learning speed and natural foraging success in bumble-bees. *Proceedings of the Royal Society B- Biological Sciences* **275**: 803–808.

Rains, G.C., Tomberlin, M., J. K.and D'Alessandro & Lewis, W.J. 2004. Limits of volatile chemical detection of a parasitoid wasp, *Microplitis croceipes*, and an electronic nose: A comparative study. *Transactions of the American Society of Agricultural Engineers* **47**: 2145–2152.

Rankin, C. 2000. Context conditioning in habituation in the nematode *Caenorhabditis elegans*. *Behavioral Neuroscience* **114**: 496–505.

Razran, G. 1971. *Mind in evolution: An East-West synthesis of learned behavior and cognition.* Houghton Mifflin, Boston, p. 430.

Reisenman, C., Insausti, T. & Lazzari, C. 2002. Light-induced and circadian changes in the compound eye of the haematophagous bug *Triatoma infestans* (hemiptera : Reduviidae). *Journal of Experimental Biology* **205**: 201–210.

Reisenman, C. & Lazzari, C. 2006. Spectral sensitivity of the photonegative reaction of the blood-sucking bug *Triatoma infestans* (Heteroptera : Reduviidae). *Journal of Comparative Physiology A-Sensory Neural and Behavioral Physiology* **192**: 39–44.

Reisenman, C., Lazzari, C. & Giurfa, M. 1998. Circadian control of photonegative sensitivity in the haematophagous bug *Triatoma infestans*. *Journal of Comparative Physiology A-Sensory Neural and Behavioral Physiology* **183**: 533–541.

Rescorla, R. 1988. Pavlovian conditioning, it's not what you think it is. *American Psychologist* **43**: 151–160.

Schilman, P. & Lazzari, C. 2004. Temperature preference in *Rhodnius prolixus*, effects and possible consequences. *Acta Tropica* **90**: 115–122.

Seidl, T. & Wehner, R. 2006. Visual and tactile learning of ground structures in desert ants. *Journal of Experimental Biology* **209**: 3336–3344.

Siwicki, K. & Ladewski, L. 2003. Associative learning and memory in *Drosophila*: beyond olfactory conditioning. *Behavioural Processes* **64**: 225–238.

Skinner, B.F. 1938. *The behavior of organisms: an experimental analysis.* Appleton-Century, Oxford, p. 457.

Srinivasan, M.V. 2010. Honey bees as a model for vision, perception, and cognition. *Annual Review of Entomology* **55**: 267–284.

Steel, C. & Ampleford, E. 1984. Circadian control of hemolymph ecdysteroid titers and the ecdysis rhythm in *Rhodnius prolixus* . *CIBA Foundation Symposia* **104**: 150–163.

Strube-Bloss, M., Nawrot, M. & Menzel, R. 2011. Mushroom body output neurons encode odor-reward associations. *Journal of Neuroscience* **31**: 3129–3140.

Sutton, M., Ide, J., Masters, S. & Carew, T. 2002. Interaction between amount and pattern of training in the induction of intermediate- and long-term memory for sensitization in *Aplysia*. *Learning & Memory* **9**: 29–40.

Takeda, K. 1961. Classical conditioned response in the honey bee. *Journal of Insect Physiology* **6**: 168–179.

Thompson, R. & Spencer, W. 1966. Habituation: A model phenomenon for the study of neuronal substrates of behavior. *Psychological review* **873**: 16–43.

Tomberlin, J., Rains, G.C. & Sanford, M.R. 2008. Development of *Microplitis croceipes* as a biological sensor. *Entomologia Experimentalis et Applicata* **128**: 249–257.

Tomberlin, J.K., Rains, G.C., Allan, S.A., Sanford, M.R. & Lewis, W.J. 2006. Associative learning of odor with food- or blood-meal by *Culex quinquefasciatus* Say (Diptera: Culicidae). *Naturwissenchaften* **93**: 551–556.

Tully, T., Bourtchouladze, R., R., S. & J., T. 2003. Targeting the creb pathway for memory enhancers. *Nature Reviews Drug Discovery* **2**: 267–277.

Tully, T., Cambiazo, V. & Kruse, L. 1994. Memory through metamorphosis in normal and mutant *Drosophila*. *The Journal of Neuroscience* **14**: 68–74.

Vaysse, G. & Médioni, J. 1976. Nouvelles experiences sur le conditionnement et le pseudoconditionnement du reflexe tarsal chez la drosophile (*Drosophila melanogaster*): Effets de chocs electriques de faible intensite. *Comptes rendus des Seances de la Societe de Biologie* **170**: 1299–1304.

Vinauger, C., Buratti, L. & Lazzari, C.R. 2011a. Learning the way to blood: first evidence of dual olfactory conditioning in a blood-sucking insect, *Rhodnius prolixus*. i. appetitive learning. *Journal of Experimental Biology* **214**: 3032–3038.

Vinauger, C., Buratti, L. & Lazzari, C.R. 2011b. Learning the way to blood: first evidence of dual olfactory conditioning in a blood sucking insect, *Rhodnius prolixus*. ii. aversive learning. *Journal of Experimental Biology* **214**: 3039–3045.

von Frisch, K. 1914. Der farbensinn und formensinn der bienen. *Zool. Jb. Physiol.* **35**: 1–188.

von Frisch, K. 1919. Uber den geruchssinn der bienen und seine blutenbiologische bedeutung. *Zool. Jb. Physiol.* **37**: 1–238.

von Frisch, K. 1967. *The dance language and orientation of bees*. Belknap Press of Harvard University Press, p. 566.

Wackers, F. & Lewis, W. 1999. A comparison of color-, shape- and pattern-learning by the hymenopteran parasitoid *Microplitis croceipes*. *Journal of Comparative Physiology A-Neuroethology Sensory Neural and Behavioral Physiology* **184**: 387–393.

Wehner, R., Boyer, M., Loertscher, F., Sommer, S. & Menzi, U. 2006. Ant navigation: One-way routes rather than maps. *Current Biology* **16**: 75–79.

Williams, A., Hollands, T. & Tucknott, O. 1981. The gas chromatographic-mass spectrometric examination in the volatiles produces by the fermentation of a sucrose solution. *Zeitschrift fur Lebensmittel-Untersuchung Und-Forschung* **172**: 377–381.

Woolhouse, M., Dye, C., Etard, J., Smith, T., Charlwood, J., Garnett, G., Hagan, P., Hii, J., Ndhlovu, P., Quinnell, R., Watts, C., Chandiwana, S. & Anderson, R. 1997. Heterogeneities in the transmission of infectious agents: Implications for the design of control programs. *Proceedings of the National Academy of Sciences of the United States of America* **94**: 338–342.

Xia, S., Feng, C. & Guo, A. 1998. Multiple-phase model of memory consolidation confirmed by behavioral and pharmacological analyses of operant conditioning in *Drosophila*. *Pharmacology Biochemistry and Behavior* **60**: 809–816.

Zeledon, R. & Rabinovich, J. 1981. Chagas-disease - An ecological appraisal with special emphasis on its vectors. *Annual Review of Entomology* **26**: 101–133.

Résumé :

Le principal objectif de ce travail de thèse est de proposer un cadre expérimental contrôlé permettant de mettre en évidence et caractériser les capacités d'apprentissage chez la punaise hématophage *Rhodnius prolixus*. Nos résultats montrent que ces insectes sont capables d'apprendre et d'associer la présentation d'une même odeur neutre avec soit la possibilité d'obtenir une récompense (un repas sanguin, conditionnement appétitif), soit avec la possibilité de recevoir une punition (un choc mécanique, conditionnement aversif). Nous avons également montré que l'apprentissage et la mémoire sont également impliqués dans le choix des hôtes. Dans un second temps, nous avons adapté à notre modèle d'étude le paradigme de conditionnement de la réponse d'extension du proboscis, ce qui a permis la caractérisation des capacités d'apprentissage, de la durée de rétention à la régulation par des horloges circadiennes. Dans son ensemble, cette étude apporte la première preuve expérimentale de la capacité d'apprentissage d'insectes vecteurs de la maladie de Chagas et propose des outils expérimentaux et méthodologiques permettant d'améliorer la compréhension des processus associés chez les insectes hématophages en général. Les résultats sont également discutés dans le contexte de la sélection d'hôte et de la transmission des parasites.

Mots clés : Apprentissage, Mémoire, Conditionnement appétitif, Conditionnement aversif, Habituation, Déshabituation, PER, Modulation Circadienne, Maladie de Chagas, Choix de l'hôte, *Rhodnius prolixus*.

Abstract :

The main goal of this work was to provide a controled experimental context allowing the strudy of learning abilities in the haematophagous bug *Rhodnius prolixus*. Our results show that these insectes are able to learn to associate the delivery of a same neutral odour either with the possibility to obtain a reward (blood-meal, appetitive conditioning) or with the possibility to receive a punishment (mechanical shock, aversive conditioning). We also showed that learning ans memory are involved in host selection processes. In a second part, we adapted to our biological model the paradigm of proboscis extension response conditioning, which allowed us to analyse and characterize its learning abilities. The maximal retention duration as well as the modulation of learning abilities by circadian clocks were evinced. Taken as a whole, this work provides the first experimental demonstration of learning abilities in Chagas disease vectors and provides experimental and methodological tools; These latters should allow improving the understanding of the mechanisms that are underlying cearning abilities of haematophagous insects in general. Results are also discussed in the context of host selection and parasite transmission.

Keywords : Learning, Memory, Appetitive conditioning, Aversive conditioning, Habituation, Dishabituation, PER, Circadian modulation, Chagas disease, Host choice, *Rhodnius prolixus*.